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"Chemical study of plants belonging to Ecuadorian flora"

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CHAPTER 1

Introduction

Chapter 1

1.6 Secondary metabolites and their role in nature

When the first so-called secondary metabolites were found, up to the 60's, they were considered as "waste products" of primary metabolism, and the accumulation of those substances in cells was attributed to the absence of an efficient excretory system in plants. However not everybody was convinced with this idea, and after some studies performed, they were describe as "trigger" substances that induce or prevent the uptake of nutrients by herbivore, as described by Ehrlich and Raven in 1964, which were the first to propose an ecological role for those substances as defense agents against insect herbivore. Research was therefore initiated in many laboratories to attempt to establish the defensive role of secondary metabolites (Harborne, 2001).

Different factors must being considered when we refer to secondary compounds, their role in plants and in nature. First of all is important to know which of those constituents is active, and how their concentration fluctuated during the life of the plant. Their localization is an important aspect.

Environmental parameters can dramatically influence the production and concentration of secondary metabolites, especially in leaf tissue; indeed we can found variations in chemistry between plants in the same population, furthermore between leaves on the same branch. The dietary impact of those compounds on the herbivore is associated to their relative solubility, so do as their relative stability within the plant and the rate of turnover.

Plant toxins are also effective against animals; an evidence is the fact that insects that feed on some species are able to assimilate certain toxins and use it to protected themselves from birds or other predators.

Moreover some of these compounds synthesized by plants are also useful to attract some kind of animals that carry out pollination and distribute the seed.

Secondary compounds also demonstrated to have a role in the interactions between plants and microorganism since both of them produce such constituents, in one case to damage the higher plant hosts, while higher plants use these substances to defend themselves from microbial infection.

Besides secondary metabolites can mediate hostile or beneficial interactions between plants growing in a given natural ecosystems. This kind of interaction is called "allelopathy".

Secondary plant constituents can be exported from the plant into the soil via the root. The concentrations thus exuded could be quite low; nevertheless they may have significant effects on bacteria and fungi that inhabit the soil around the plant roots.

One way in which a higher plant might reduce the "metabolic costs" of synthesizing and storing toxins is to produce the defense agent only when it is actually needed.

Toxins needed for protection are storage, in vacuoles in most of the cases, in a safe inactive bound form in plants, for example attached to a sugar.

1.7 Use of medicinal plants in Ecuador

About 80 % of the population of Ecuador depends on the traditional medicine for its health, thus on plants or natural products (Buitrón, 1999). This tendency is growing due to different reasons as for example the difficulties that population has to access to medical attention based on commercial drugs and to medical social security. The use of traditional medicine becomes everyday more common not just in rural areas, by indigenous people, or in poor urban neighborhoods, but in general as a result of the search for effectiveness, reduction of harmful practices in the treatment of illnesses, low costs and to avoid collateral effects caused sometimes by some chemicals products.

Nevertheless most part of the species from Ecuador have not been identified, selected or test in an efficiently way, in order to be include in the national list of drugs, leading to a lack of stimulus for their production and appropriate use. It must be more interest to encourage research, to develop management and conservations programs that promote their agronomic production, chemical study and industrialization.

In addition, as it happens with commercial drugs, the indiscriminate use and the self-medication with natural products cause problems, since in Ecuador is very easy to find and buy medicinal plants or natural products derived from them, never tested before in a scientific way and without any quality control, in urban or rural markets, handicraft, and pharmacy stores. The fact that appropriated standardization process and good practice of production are not applied increase the risk of the use of natural products. These kinds of difficulties bring to the lost of the knowledge and the resources.

Ecuador has a very rich biodiversity, but from all the vast number of species corresponding to medicinal plants only 500 are known. For the most part of these species there is not enough scientific information or an ecological profile that could help to find the best conditions to obtain and produce sufficient raw material to satisfy the existing demand.

Plants growing in the zone of the Cordillera de los Andes, where the species concerning this work has been collected, are the better known and the most demanded (Buitrón, 1999).

Most of the time the collection of the species is carried out directly by consumers, as in the case of indigenous people for daily consumption. The whole plant could be used or commercialize as raw material, or in parts, pulverized, as extracts or as semi-synthetic substances. People use medicinal plants for infusions, to treat different sickness as headache, back pain, skin illness, or as antibiotics, antifungal, among others.

This is why it becomes indispensable to improve the research in this field in Ecuador, to conserve the species and their habitats, improving the use and trade of medicinal plants and derivative products, in order to benefit the population's health. Is important to enrich the knowledge about the chemical content of these species, to reach a better understanding about their properties and encourage an appropriate consumption and use of natural products.

1.8 Bioactive compounds from natural sources and drug discovery

The evolution of secondary metabolites has resulted in diverse, more or less complex natural chemicals able to interact with a variety of targets from other organisms and environment. The biosynthesis and selection of these chemical entities enhance organisms

survival and competitiveness. Their bioactive nature and diversity render these chemical compounds the most profitable and efficient source of new drug leads.

Natural product preparations, mainly herbal remedies, have historically been the major source of pharmaceutical agents. Even today about 70 % of the world´s population relies upon medicinal plants for its primary pharmaceutical care (Rosen *et al*., 2009).

Natural product research is a demanding task in the sense of supply of natural material, analytics, reproducibility, isolation procedure, structure elucidation and chemical complexity.

Clues for an idea about the bioactivity of a novel natural compound may be provided from the interacting ecosphere itself, for example, microorganism or herbivores that can be repelled successfully, or animals are observed to their exposure to natural materials and their subconscious, instinctive or conscious application to treat and cure impairments.

The advancement in bioassay technology and high throughput screening has perhaps been one of the strongest factors for the reemerging role of natural products in drug discovery and development over the last decades (Rosen *et al*., 2009). Nevertheless, nowadays it remains a challenge, in the analysis and evaluation of the obtained results, to understand more about their activity, depending on lot of parameters as concentration, additive and synergistic effects, as well as assay or agents that could disturb metabolites activity.

Nowadays natural product compounds become the source of numerous therapeutic agents. Compounds with an important activity to treat cancer, resistant bacteria and virus, so as immunosuppressive disorders have been developed thanks to recent progress in techniques directed to discover drugs from natural product sources (Gullo *et al*., 2006).

In the areas of cancer and infectious disease, 60 and 75% respectively, of new drugs, originate from natural sources. Whereas compounds derived from natural products represent 78% of commercial antibacterials (Gullo *et al*., 2006). Nevertheless there is a continuing need to discover novel active substances since antibiotic resistance by pathogens limit the efficiency of marketed products.

One of the main challenges in this field is how to determine desirable biological activity. In the beginning empirical observations by native people allowed to have an idea about which plant and animal products were effective for a variety of diseases. Through the years a new

process with more scientific basis, known as screening, took place. It consists on search compounds with a predefined set of biological activities by surveying as much diversity as it is possible. Nevertheless considering the vast number of species of plants in nature, besides the fact that just a very small part of those have been evaluated for their biological activity, it would take too much time to reach the goal. Thus a second approach very useful is the one we mentioned before, that is to control the information provided by indigenous cultures about the uses they give to natural products to prevent or treat diseases. This discipline known as ethnopharmacology, is a mix of sociology, medicine, anthropology and botany (Clark, 1996).

These cultures have continued to use vegetal material for very long time because of its effectiveness. Hence the need to record and validate this knowledge, as it is passed from generation to generation orally and there is a risk to lose it. However some cultures registered in written from the information. It is the case of the Chinese "Materia Medica", where are reported details of the classification, description, preparation and uses of medicinal plants.

In order to find potential lead compounds by a phytochemical study, the first step is the choice of the plant material on the basis of chemotaxonomic and phylogenetics features, ethnobotanic and/or other literature information.

Generally, since the starting point is a heterogeneous extract, it is necessary the fractionation and identification of the bioactive components carried out often with the help of biological assays. Then the pure compounds have to be characterized. The process to obtain pure molecules from the natural source is complex and usually yields small amounts of the product. Many extraction and chromatographic techniques are required, and the characterization is obtained thanks to nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis. The method is described in **Figure 1.1**.

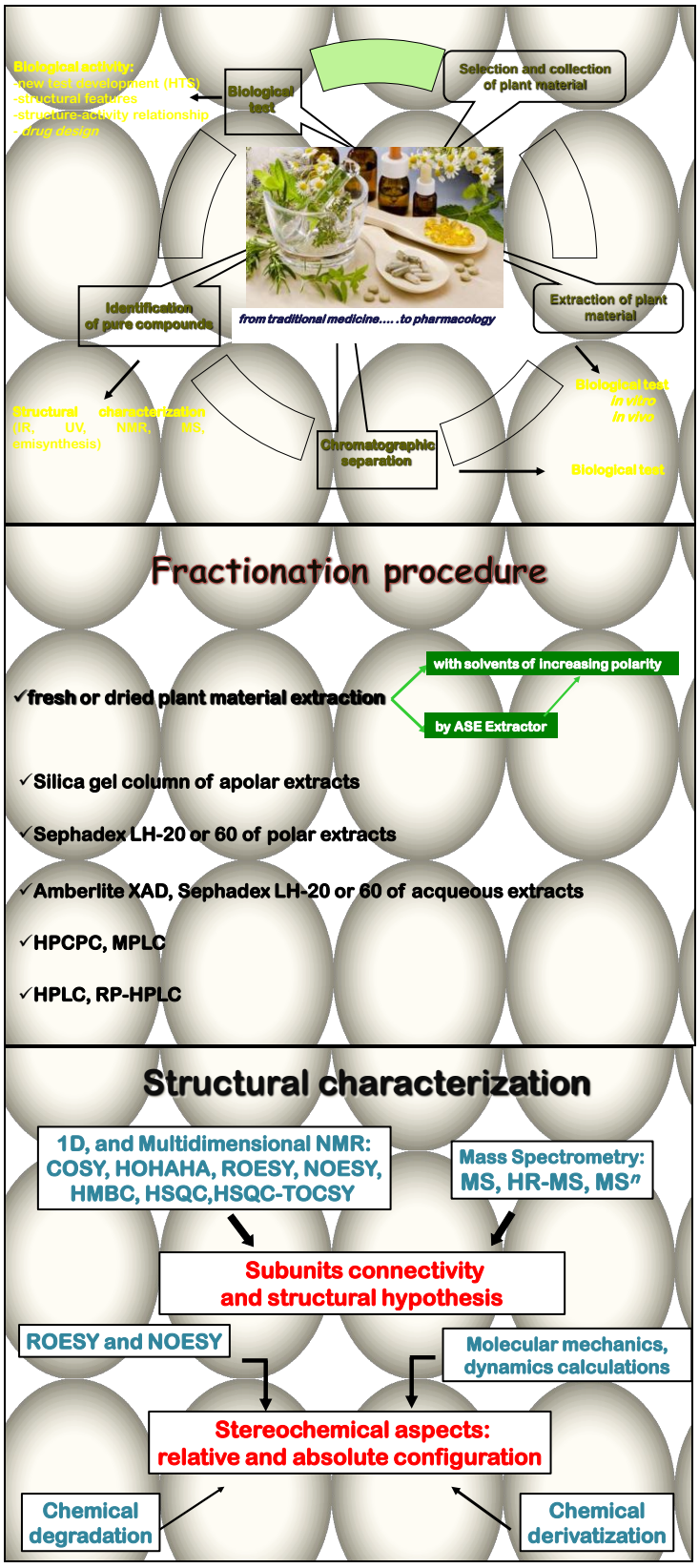


Figure 1.1*: Compounds isolation and structural characterization scheme*

1.9 The aim of the thesis project

The title of my PhD thesis is "**Chemical study of plants belonging to Ecuadorian flora**".
I performed my research at the Dipartimento di Farmacia of the University of Pisa, working with the phytochemical group under the supervision of Prof. Alessandra Braca. Among all the projects in this research laboratory, there is the investigation of plants growing in the developing countries of South America, Africa, Middle East and Asia.

The aims of my research were:
To isolate and characterize bioactive compounds from medicinal plants collected in Ecuador following this experimental protocol:
-Selection of the plant
-Extraction from the natural source
- Isolation of pure compounds
-Structural characterization
-Elucidation of biological activity
To discover biologically active plants from Ecuador that could be useful in the medical field, starting from the knowledge about their use as natural remedies in the traditional medicine, in order to find scientific arguments that support these uses.
To show the importance of this kind of research, mainly in countries as Ecuador with such rich biodiversity, not just to contribute with the scientific community but giving resources that could provide scientific material to encourage the research and the development in the country and improve quality of life and health.

In this framework I studied five species that have not been studied before for their chemical content despite their use in traditional medicine, and that belong to interesting plant families:
Siparuna thecaphora (Poepp. Et Endl) A.DC. (Monimiaceae), *Bidens humilis* (Kunth)

(Asteraceae), *Andromachia igniaria* (Humb.& Bonpl.) (Asteraceae), *Euphorbia laurifolia* (Juss. Ex Lam.) (Euphorbiaceae), and *Clinopodium tomentosum* (Kunth) Harley (Lamiaceae).

The work on all plants deals principally with the isolation and structural characterization of secondary metabolites by using different chromatographic separation technique, NMR spectroscopy, and mass spectrometry. For two species different test were carried out to analyses the specific biological activity of the samples.

1.10 Content of the thesis

The results of the research on *Siparuna thecaphora, Clinopodium tomentosum, Euphorbia laurifolia*, *Bidens humilis* and *Andromachia igniaria* are reported in the **Chapters 3** to **7**, with all the new and known compounds isolated and characterized through chromatographic, spectrometric and spectroscopic techniques. In the case of *E. laurifolia* and *B. humilis* different biological test were applied to a pure new compound in the first case, and to the extracts and to the new and known isolated compounds obtained in the case of the second species. The results and details are reported in their respective chapters.

Conclusions of the thesis are finally reported in a unique session.

CHAPTER 2

Materials and methods

Chapter 2

2.1 Chromatographic techniques

2.1.1 Thin Layer Chromatography (TLC)

TLC (**Figure 2.1**) was performed on precoated kieselgel 60 F_{254} plates (Merck), 0.25 mm thick, with glass or aluminium as support. The spots were revealed using UV detection with lamp at 254 or 366 nm and successively using a specific spray reagent, allowing to the development of typical colouring that can give information about the nature of examined compounds. As eluting solvents in the TLC analyses, some mixtures of solvents were mainly used: $CHCl_3$:MeOH (99:1), $CHCl_3$:MeOH (98:2), $CHCl_3$:MeOH (97:3), $CHCl_3$:MeOH (95:5), $CHCl_3$:MeOH (9:1), $CHCl_3$:MeOH:H_2O (40:9:1), $CHCl_3$:MeOH:H_2O (70:30:3), *n*-BuOH:AcOH:H_2O (60:15:25).

$CeSO_4/H_2SO_4$, a general reactive, was used to reveal all compounds contained in mixtures. It is a saturated solution of cerium sulphate in 65% sulphuric acid. Heating at 120°C for 15 min is needed to reveal the spots.

Other reactives were also used in this work:

NTS/PEG: adopted commonly for polyphenolic compounds such as flavonoids or coumarins. It corresponds to a mixture of the following solutions:

Naturstoff reactive (NTS) = β-ethylaminic diphenylboric ester in methanol solution 1% and Polyethylene glycol 4000 (PEG) in ethanol solution 5%.

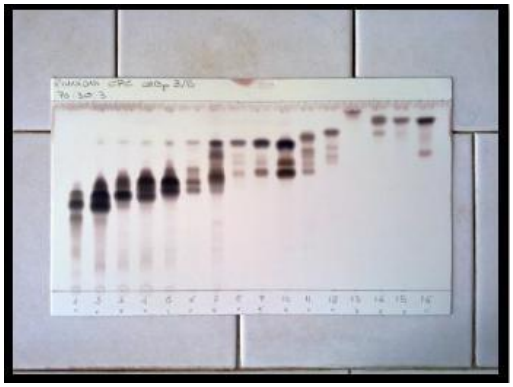


Figure 2.1: *TLC*

2.1.2 Column Chromatography

2.1.2.1 Gel Filtration Chromatography

Gel filtration chromatography was performed over Sephadex LH-20 (25-100 μm, Pharmacia Fine Chemicals) using columns 3 x 100 cm, for 2-4 g of extract, and 5 x 100 cm for 5-10 g of material, and a peristaltic pump Pharmacia Fine Chemicals P1. The eluent was always methanol, at constant flow rate (0.8 mL/min).

2.1.2.2 Flash Chromatography

Some separation were carried out with Biotage® Isolera® Spektra (**Figure 2.2**) for flash purification system to improve fractions and compound purity with λ detection and PDA spectral analysis at 254 and 320 nm. Cartridge size, related to the quantity of the mixture charged, is reported in **Table 2.1**.

Figure 2.2: *Biotage® Isolera® Spektra*

Table 2.1. *Biotage® Isolera® parameters*

Cartridge (g)	Load capacity (mg)	Flow rate (ml/min)
10	100-500	10-20
25	250-1250	15-40
50	500-2500	30-50
100	1000-5000	30-50
340	3400-17000	65-120

2.1.2.3 High Performance Liquid Chromatography (HPLC)

Reverse-phase HPLC separations (**Figure 2.3**) were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C_{18} µ-Bondapak column (30 cm x 7.8 mm, 10 µm Waters, flow rate 2.0 ml/min). MeOH:H_2O mixtures were used as mobile phases.

Figure 2.3: *HPLC system*

2.1.2.4 High Performance Centrifugal Chromatography (HPCPC)

HPCPC was carried out on a EverSeiko CPC240 apparatus (**Figure 2.4**) equipped with 3136 cells (ϕ 15 mm and 240ml), Eluting with mixtures of solvents accurately selected on the basis of chemical composition of sample.

Figure 2.4: *HPCPC system*

2.2 Nuclear Magnetic Resonance analyses (NMR)

NMR experiments were performed on Advance Bruker 250 spectrometer (**Figure 2.5**) and Bruker DRX-600 spectrometer at 300 K. All the 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI

(Time Proportional Phase Increment) used to achieve frequency discrimination in the ω_1 dimension.

Standard pulse sequences and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, and ROESY experiments. NMR data were processed on using xwin-NMR software.

Chemical shifts were expressed in δ (parts per million) referred to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD.

Figure 2.5: *Advance Bruker 250 NMR spectrometer*

2.3 Mass Spectrometry analyses (MS)

MS and MS^n analyses (positive and negative mode) were obtained using a LCQ Advantage spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ion trap analyzer and Xcalibur 3.1 software (**Figure 2.6**). All the analyzed molecules were dissolved in MeOH and then diluted to 10-20 μm/mL. Samples were injected into the spectrometer at a flow rate of 5 μL/min using an external syringe pump.

HRESIMS were achieved using a nano LC-MS/MS system, with a nanoAcquity UPLC module and a Q-TOF premiere spectrometer equipped with a nanoelectrospray ion source (Waters-Milford, MA, USA), and provided with a lock-mass apparatus to perform a real-time calibration correction.

Liquid chromatography separation of *Bidens humilis* extracts were achieved using a Luna C_{18} (150 x 2.1 mm, 2.5 µm Phenomenex, Palo Alto, USA) and 0.1% formic acid in water (A) and acetonitrile (B). Compounds elution was performed using a linear gradient from 1 % to 50 % of B in 45 minutes. Mass spectra were acquired both in positive and in negative ion mode, over the 200-800 *m/z* range. Source temperature was set at 120 °C, capillary voltage 3300 eV, and cone voltage 50 eV. HRESIMS/MS spectra were acquired for the three most abundant ion observed in each MS spectrum (dependent scan mode). When required, HRESIMS/MS spectra were acquired, setting specific parent ion in the mass spectrometry method.

Figure 2.6: *Mass spectrometer*

2.4 Optical Rotation analyses

Optical rotations analyses were measured on a Perkin–Elmer 241 polarimeter (**Figure 2.7**) equipped with a sodium lamp (589 nm) and a 1 dm microcell.

Figure 2.7: *Polarimeter*

2.5 Ultraviolet-Visible (UV) Spectroscopy

UV spectra were recorded on a Perkin–Elmer–Lambda Spectrophotometer (**Figure 2.8**). Samples were disolved in MeOH.

Figure 2.8: *Spectrophotometer*

2.6 Acid Hydrolysis

A solution of each compound (2.0 mg) in 1 N HCl (1 ml) was stirred at 80 ºC in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl) imidazole and pyridine (0.2 ml), and the solution was stirred at 60 ºC for 5 min. After drying the solution, the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm x 25 m). Temperatures of both the injector and detector were 200 ºC. A temperature gradient system was used for the oven, starting at 100 ºC for 1 min and increasing up to 180 ºC at a rate of 5 ºC/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-xylose, L-arabinose, L-rhamnose, and D-glucose (Sigma–Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

2.7 Biological activity

2.7.1 Drug Affinity Responsive Target Stability (DARTS)

Drug Affinity Responsive Target Stability (DARTS) is a general methodology for identifying and studying protein-ligand interactions. The technique is based on the principle that when a small molecule compound binds to a protein, the interaction stabilizes the target protein's structure such that it becomes protease resistant. DARTS is particularly useful for the initial identification of the protein targets of small molecules, but can also be used to validate potential protein-ligand interactions predicted or identified by other means and to estimate the affinity of interactions. The approach is simple and advantageous because it can be performed using crude cell lysates or other complex protein mixture (without requiring purified proteins), and uses native, unmodified small molecules (Lomenick *et al.*, 2011).

2.7.1.1 Cell lysates

Human prostate cancer (PC3) and human breast adenocarcinoma (MCF7) cell lines were lysed in PBS containing 0.1% Igepal (lysis buffer) and a protease and phosphatase inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was determined by Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine albumin as standard. Lysates (50 μg) were incubated with 5 μl of PBS 8.5% DMSO or 5 μl of compound **4** of *Euphorbia laurifolia* 200 μM 8.5% DMSO in PBS, to obtain the final concentration of 20 μM 1% DMSO for each small molecule studied. The samples were incubated for 1 hour at 4 °C under stirring. Samples were then underwent proteolysis with subtilisin (enzyme: lysate 1:750 w/w for 60 min at 30 °C) followed by 10% SDS-PAGE analysis. In fact, to stop proteolysis, 5 μl of Laemmly Buffer 4X was added to each sample and incubated at 100 °C for 2 min.

2.7.1.2 Gel digestion

In gel digestion by trypsin was performed as described in Dal Piaz *et al.*, 2013. Briefly, the lane of interest were excised manually and analyzed as follow: gel pieces were discolored and de-hydratated in CH_3CN. Subsequenty, samples were subjected to reduction in 10 mM

dithiothreitol (DTT) for 1 hour at 56 °C and alkylated in 55 mM iodoacetamide (IAA) for 30 min at r.t. in the dark. Samples were washed again, with CH_3CN and ammonium bicarbonate 100 mM and finally digested with 30 µl of trypsin, from porcine pancreas (SIGMA) at a final concentration of 0.013 ng/µl. Supernatans were collected, vacuum dried, dissolved in 15 µl 5% formic acid for MS analysis (Dal Piaz *et al.,* 2013).

2.7.2 Chemicals

Sodium acetate trihydrate, DPPH, 2,4,6-tripyridyl-s-triazine (TPTZ), Iron (III) chloride ($FeCl_3*6H_2O$), β-carotene, linoleic acid, Tween 20, butylhydroxytoluen (BHT), Folin-Ciocalteu reagent, sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were purchased from Sigma-Aldrich (Milano-Italy). *n*-Hexane, chloroform, methanol, hydrochloric acid and glacial acetic acid were purchased from VWR (Milano-Italy).

2.7.3 DPPH radical-scavenging activity

The ability to scavenge the DPPH free radical was monitored according to the method reported by Milella *et al*. (2014) with slight modifications. All samples were tested individually at different concentrations by addition to a methanol solution of DPPH radical (100 µM). For each measure 50 µL appropriately diluted sample was added to 200 µL of DPPH reagent, the mixtures were stirred and allowed to stand in the dark at room temperature. In the control 50 µL of methanol, instead diluted sample was added to 200 µL of DPPH solution. The absorbance of the resulting solutions was measured at 515 nm after 30 min. In all experiments Trolox radical scavenging activity was also determined and used as a reference. Sample activity was expressed as mg of Trolox equivalents per gram of sample (mg TE/g). Each reaction was performed in triplicate and results expressed as mean ± standard deviation (Padula *et al*., 2013).

2.7.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as described by Russo *et al.* (2012) with slight modifications. The FRAP reagent was made fresh before each experiment and it was prepared by mixing 300 mM acetate buffer in distilled water pH 3.6, 20 mM $FeCl_3*6H_2O$ in distilled water and 10 mM TPTZ in 40 mM HCl in a proportion of 10:1:1. For each sample 25 µL of appropriately diluted sample (25 µL of methanol for the blank) and 225 µL of FRAP reagent was added and incubated at 37°C for 40 min in the dark. Absorbance of resulting solution was measured at 593 nm. Trolox was used as a reference antioxidant standard and FRAP values were expressed as mg Trolox equivalents per gram of sample (mg TE/g). Each reaction was performed in triplicate and results expressed as mean ± standard deviation (Russo *et al*., 2012).

2.7.5 β-Carotene bleaching (BCB) assay

For this assay the following reagents were mixed: β-Carotene solution (0.2 mg of β-carotene dissolved in 0.2 mL of chloroform), linoleic acid (20 mg) and Tween 20 (200 mg). Chloroform was removed by using rotary evaporator at room temperature (Russo *et al*., 2012). Distilled water (50 mL) was added with oxygen, then 950 µL of the emulsion were transferred into several tubes containing 50 µL of sample (the final concentration for all tested samples was 0.05 mg/mL) or methanol as blank. BHT was used as positive control. Next, 250 µL of emulsion-sample solution was transferred (250 µL/well) to the reaction plate. Since the reaction was temperature-sensitive, close temperature control throughout the plate was essential in this assay; therefore outer wells were filled with 250 µL of water to provide a large thermal mass (Mikami *et al*., 2009). The microplate was immediately placed at 50°C for 3 h and the absorbance was measured at 470 nm at 0', 30', 60', 90', 120', 150', and 180'. Each sample was carried out in triplicate and results expressed as mean ± standard deviation. Results were expressed as percentage of BCB inhibition and calculated as follows: $(A_{\beta\text{-carotene after 180 min}}/A_{\text{initial }\beta\text{-carotene}})$ x 100 (AA%).

2.7.6 Total polyphenolic content (TPC)

TPC was determined according to the Folin-Ciocalteu method (Milella *et al*., 2014) by adding 75 µL of the diluted samples extract (in the blank 75 µL of methanol) to 425 µL of distilled water, 500 µL of Folin-Ciocalteu reagent and 500 µL of a sodium carbonate aqueous solution (10% w/v). The mixture was stirred and left in the dark at room temperature for 60 min then absorbance was measured at 723 nm. Gallic acid was used as reference standard and TPC was expressed as mg Gallic Acid Equivalents (mgGAE)/g of sample. All analyses were performed in triplicate and results expressed as mean ± standard deviation.

CHAPTER 3

Phytochemical study of *Siparuna thecaphora* (Poepp. et Endl.) A. DC.

Chapter 3

3.1 Introduction: *Siparuna* genus

The genus *Siparuna* (Siparunaceae, formerly placed in Monimiaceae family) comprises about 150 species of shrubs, small trees or climbers that inhabit tropical regions of Mexico, West Indies, Central and South America (Renner *et al*., 1997). Various species have been used in traditional medicine for the treatment of gastrointestinal disorders, skin diseases, snakebites, fever, colds, headache and rheumatism (Leitao *et al*., 2000). In Latin America, *Siparuna* species are also used by traditional healers from different countries for some rituals or to treat malaria.

Phytochemical studies were carried out previously on some Central American species of *Siparuna* concerning benzylisoquinoline alkaloids, terpenoids and flavonoids (Leitao *et al*., 1999; Jenett-Siems *et al*., 2003). *Siparuna thecaphora* (Poepp. et Endl.) A. DC. is an aromatic shrub or small tree with opposite, simple leaves and unisexual flowers; the fruits are strong lemon scented, such that it is locally called 'limoncillo'. Due to its highly variable morphology, a great number of synonyms are found in the literature (e.g. *Siparuna nicaraguensis* Hemsl., *Siparuna andina* (Tul.) A.DC. and *Siparuna gilgiana* Perkins). Some chemical studies of *S. thechaphora* have been done previously: alkaloids were isolated from the twigs (under the synonym *S. nicaraguensis*) (Gerard *et al*., 1986) and the roots (under the synonym *S. gilgiana*) (Chiu *et al*., 1982). However, except for the composition of the essential oil (Vila *et al*., 2002), there is no information concerning the composition of secondary metabolites of the leaves.

Figure 3.1: *Siparuna thecaphora*

3.2 Plant material

Leaves of *S. thecaphora* were collected in Puerto Napo, Ecuador in June 2007. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador, where a voucher specimen was deposited (number MO-256138).

3.3 Extraction and isolation

The dried and powdered leaves (310 g) of *S. thecaphora* were successively extracted for 48 h with *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1) and MeOH, by exhaustive maceration (3 X 2L), to give 13.3, 7.4, 4.5 and 10.6 g of the respective residues.

The $CHCl_3$-MeOH extract (4.5 g) was subjected to CC over Sephadex LH-20 in MeOH to give eight major fractions (A-H) grouped by TLC. Fractions B (123 mg) and D (141 mg) were separately purified by reverse-phase high performance liquid chromatography (RP-HPLC) with MeOH-H_2O (2:3) as eluent to afford a new compound *trans*-thujane-1α,7-diol 1-*O*-β-D-glucopyranoside (compound **1**, 2.7 mg, t_R = 24 min) from fraction B and 3,4-dihydroxybenzaldehyde (compound **2**, 8.4 mg, t_R = 8 min) from fraction D, respectively. Fraction G (91 mg) was also purified by RP-HPLC with MeOH-H_2O (4.5:5.5) as eluent to yield quercetin 3-*O*-β-D-glucopyranoside (compound **3**, 8.4 mg, t_R = 16 min). The MeOH

extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH residue (2.7 g). The *n*-BuOH fraction (2.7 g) was submitted to a Sephadex LH-20 CC using MeOH as eluent to obtain six major fractions (A-F) grouped by TLC. Fractions C (118 mg) and E (34 mg) were purified by RP-HPLC with MeOH-H_2O (4.5:5.5) as eluent to give rutin (compound **4**, 4.5 mg, t_R = 17 min) from fraction C and quercetin 3-*O*-β-D-galactopyranoside (compound **5**, 8.5 mg, t_R = 16 min) from fraction E, respectively.

The structure of new compound **1** is show in **Figure 3.2**, the structure of the other compounds **2-4** are reported in **Figure 3.3**.

3.4 New isolated compounds

Trans-thujane-1α,7-diol 1-*O*-β-D-glucopyranoside (**1**).

Yellow oil $[\alpha]_D$ = -126 (c 0.05, MeOH); ^{1}H NMR data (600 MHz, CD_3OD, δ in ppm, *J* in Hz): 0.55 (1H, dd, *J* = 8.0 and 3.5 Hz, H-3a), 0.92 (3H, d, *J* = 6.5 Hz, Me-9), 0.95 (3H, d, *J* = 6.5 Hz, Me-10), 0.99 (1H, dd (C-2_{glc}), 77.8 (C-3_{glc}), 77.9 (C-5_{glc}), 67.5 (C-7), 91.0 (C-1), 100.2 (C-1_{glc}). HR-ESI-MS: *m/z* 333.1897, *J* = 5.0 and 3.5 Hz, H-3b), 1.28 (1H, dd, *J* = 8.0 and 5.0 Hz, H-2), 1.37 (1H, septet, *J* = 6.5 Hz, H-8),1.58 (1H, ddd, *J* = 16.0, 5.5 and 5.5 Hz, H-6a), 1.60 (1H, ddd, *J* = 14.0, 8.5 and 5.5 Hz, H-5b), 1.63 (1H, ddd, *J* = 14.0, 5.5 and 5.5 Hz, H-5a), 1.73 (1H, ddd, *J* = 16.0, 8.5 and 5.5 Hz, H-6b), 3.22 (1H, dd, *J* = 9.0 and 8.0 Hz, H-2_{glc}), 3.29 (1H, m, H-5_{glc}), 3.31 (1H, t, *J* = 9.0 Hz, H-4_{glc}), 3.40 (1H, t, *J* = 9.0 Hz, H-3_{glc}), 3.56 (2H, s, H_2-7), 3.66 (1H, dd, *J* = 12.0 and 5.0 Hz, H-$6a_{glc}$), 3.82 (1H, dd, *J* = 12.0 and 3.5 Hz, H-$6b_{glc}$), 4.65 (1H, d, *J* = 8.0 Hz, H-1glc). ^{13}C NMR (600 MHz, CD_3OD, δ in ppm, *J* in Hz): 12.8 (C-3), 19.8 (C-9), 19.9 (C-10), 25.4 (C-5), 26.6 (C-2), 29.9 (C-6), 33.4 (C-4), 33.7 (C-8), 62.7 (C-6_{glc}), 71.5 (C-4_{glc}), 75.0 (C-2glc), 77.8 (C-3glc), 77.9 (C-5glc), 67.5 (C-7), 91.0 (C-1), 100.2 (C-1glc). HR-ESI-MS: *m/z* 333.1897 $[M + H]^+$ (calcd for $C_{16}H_{29}O_7$, 333.1913); ESI-MS: *m/z* 355 $[M + Na]^+$, 193 $[M + Na - 162]^+$.

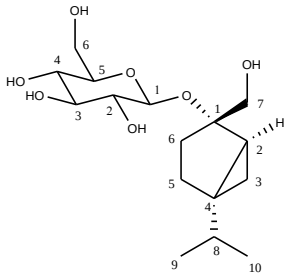


Figure 3.2: New isolated compound from *S. thecaphora*

3.5 Structural elucidation

As described before chromatographic separation of chloroform-methanol and methanol extracts of *S. thecaphora* leaves led to the isolation of one new monoterpene glycoside (**1**) (**Figure 3.2**) and four known compounds, namely 3,4-dihydroxybenzaldehyde (**2**) (Malarczyk *et al.*, 1994), quercetin 3-O-β-D-glucopyranoside (**3**), quercetin 3-*O*-β-D-galactopyranoside (**5**) and rutin (**4**) (Agrawal, 1989). The structures of all the known compounds were determined by spectral analysis and comparison of data with those reported in the literature.

Compound **1**, a yellow oil, showed a quasi molecular ion peak at *m/z* 355 $[M+Na]^+$ in the positive ESI-MS spectrum. The molecular formula was established to be $C_{16}H_{28}O_7$ by HR-ESI-MS and ^{13}C NMR analyses. In the MS^2 spectrum, a prominent fragment at *m/z* 193 $[M + Na - 162]^+$ was observed, due to the loss of one hexose residue. Its ^1H NMR spectrum showed the presence of a monoterpene structure (Jakupovic *et al.*, 1991) with two methyl doublets [δ 0.92 and (d, J = 6.5 Hz)] one hydroxymethylene group (δ 3.56, s), three methylenes (one of them was considered to be a methylene of a cyclopropane ring at δ 0.55, dd, J = 5.0 and 3.5 Hz), two methines and protons ascribable to a monosaccharide group. The ^{13}C NMR of **1** revealed the presence of two methyls (δ 19.8 and 19.9), four methylenes, of wich one was oxygenated (δ 12.8, 25.4, 29.9 and 67.5), two quaternary carbons (δ 33.4 and 91.0) and characteristics signals of a β-glucopyranosyl group (δ 62.7, 71.5, 75.0, 77.8, 77.9 and 100.2). Assignments of all chemical shifts of protons and carbons were ascertained from a combination of 1D total

correlation spectroscopy (TOCSY), double-quantum filtered correlated spectroscopy (DQF- COSY), heteronuclear single quantum coherence (HSQC) analysis (Sanogo *et al.*, 1998). Direct evidence of the substituent sites was derived from the HSQC and heteronuclear multiple bond coherence (HMBC) data. The HMBC correlations between the proton signal at δ 3.56 (H_2-7) and the carbon resonances at δ 26.6 (C-2), 29.9 (C-6) and 91.0 (C-1); between the proton signal at δ 1.73 (H-6b) and the carbon resonances at δ 26.6 (C-2), 33.4 (C-4), 67.5(C-7) and 91.0 (C-1); between the proton signal at δ 1.28 (H-2) and the carbon resonances at δ 33.4 (C-4) and 67.5 (C-7)and between the proton signal at δ 0.99 (H-3b) and the carbon resonances at δ 25.4 (C-5), 33.7 (C-8) and 91.0 (C-1) substantiated the presence of hydroxyl groups at C-1 and C-7, while correlations between δ 4.65 (H-1_{glc}) and 91.0 (C-1) confirmed the position of the glucopyranosyl moiety. The configuration of the glucopyranosyl moiety was determined to be D by hydrolysis of **1**, trimethylsilysation and gas chromatography (GC) analysis. The relative stereochemistry of **1** was ascertained by 2D rotating frame Overhauser effect spectroscopy experiment. Rotating Overhauser effect correlations were observed between H-1_{glc} and H-2 and between Me-10 and H-2 and H-3a. On the basis of all NMR evidences, the structures of **1** were determined as thujane-1α, 7-diol 1-*O*-β-D-glucopyranoside.

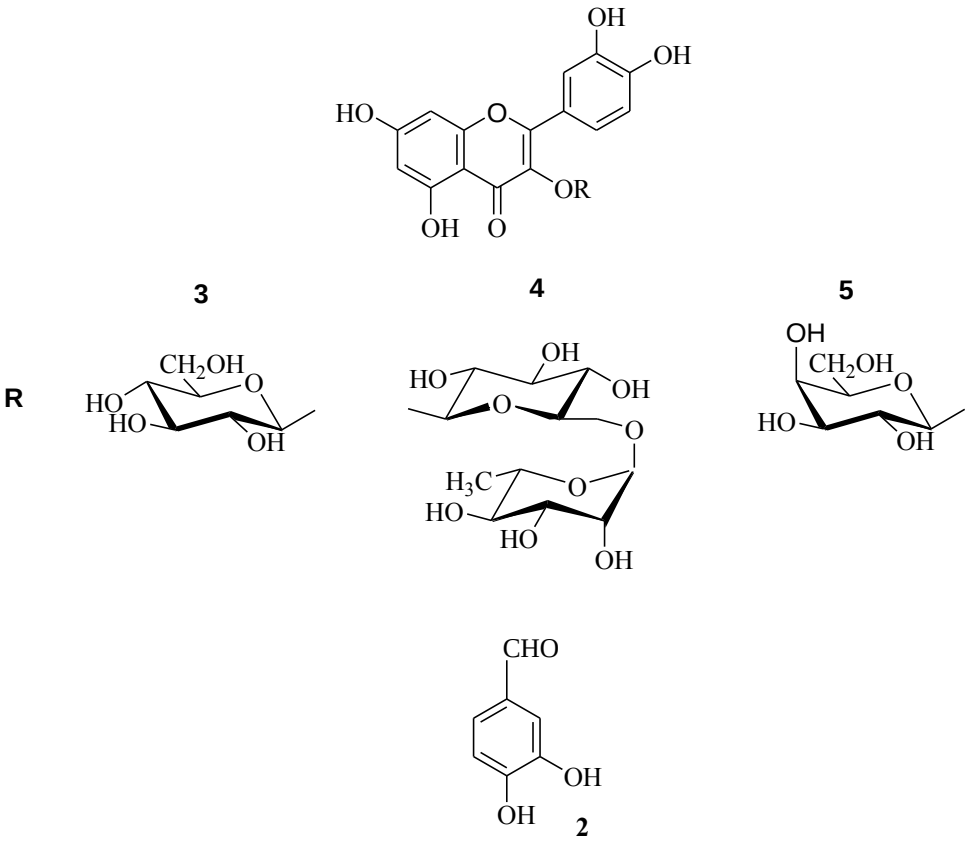


Figure 3.3: Structures of known compounds from *S. thecaphora*

CHAPTER 4

Phytochemical study of *Clinopodium tomentosum* (Kunth) Govaerts

Chapter 4

4.1 Introduction: *Clinopodium* genus

The genus *Clinopodium* (Lamiaceae) consists of flowering plants, wildly distributed in southern and southeastern Europe, in the North America and Mexico (Estrada-Reyes *et al*., 2010). It is also found growing in Latin America between 3000 and 4000 m a.s.l. Many species of the genus are used as medicinal plants. *Clinopodium tomentosum* (Kunth) Govaerts (**Figure 4.1**) possesses small yellow-colored flowers, reaching a height of 30–80 cm and in Ecuador is commonly known as "Santa Maria". Local people use the aerial parts of the plant to prepare infusions for its relaxant effect and as anti-inflammatory agent. Previous phytochemical studies on *Clinopodium* ssp. have revealed the presence of flavonoid glycosides, phenylpropanoids, caffeic acid oligomers, and saponins (Miyase and Matsushima, 1997; Opalchenova and Obreshkova, 1999; Murata *et al.*, 2009; Aoshima *et al.*, 2012). Despite its use in the Ecuadorian traditional medicine, to our knowledge, no data on the chemical composition or biological activity of the aerial parts of *C. tomentosum* are available. Nevertheless, its essential oil composition was reported by Benzo *et al*. in 2007. Next we reported the isolation and structural characterization by spectroscopic and spectrometric methods of one new compound, named 2-*O*-benzoyl-3-*O*-cinnamoyl tartaric acid (**1**) (**Figure 4.2**) along with twelve known compounds, from the aerial parts of the title plant.

Figure 4.1: *Clinopodium tomentosum*

4.2 Plant material

Aerial parts of *C. tomentosum* were collected in Tumbaco, Ecuador in September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 7305 Clinopodium tomentosum/1) was deposited at Herbarium Horti Botanici Pisani, Pisa, Italy.

4.3 Extraction and isolation

The dried and powdered aerial parts (560 g) of *C. tomentosum* were in sequence extracted for 48 h with *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1) and MeOH, by exhaustive maceration (3 x 2 L), to give 7.6, 18.0, 8.5 and 13.1 g of the respective residues. The $CHCl_3$-MeOH extract (2.5 g) was subjected to Sephadex LH-20 (CC, 3 x 70 cm, flow rate 1.5 mL/min) eluting with MeOH to give six major fractions (A-F) grouped by TLC, together with pure caffeic acid (compound **2**, 50 mg, 370-420 mL) and hesperitin (compound **3**, 40 mg, 560-1000 mL). Fractions C (328 mg), D (904 mg), and E (157.8 mg) were separately purified by RP-HPLC with MeOH-H_2O (2:3) as eluent to afford blumenol c glucoside (compound **4**, 5.3 mg, t_R = 17 min) from C, syringaresinol 4'-*O*-β-D-glucopyranoside (compound **5**, 8.6 mg, t_R = 17 min) and dihydrodehydroconiferyl alchol 9'-*O*-β-D-glucopyranoside (compound **6**, 10 mg, t_R = 19 min) from D, and *p*-coumaric acid (compound **7**, 6 mg, t_R = 16 min), caffeic acid methyl ester (compound **8**, 10 mg, t_R = 24 min) and caffeic acid ethyl ester (compound **9**,7.7mg, t_R = 42 min) from E. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH residue (6.2 g). The *n*-BuOH fraction (6.2 g) was submitted to Sephadex LH-20 (CC, 5 x 70 cm, flow rate 1.5 mL/min) using MeOH as eluent to obtain seven major fractions (A-G) grouped by TLC, together with pure caffeic acid (compound **2**, 14.2 mg, 260-270 mL) and clinopodic acid E (compound **10**, 62.4 mg, 550-590 mL). Fractions D (135.5 mg) and E (243 mg) were separated by RP-HPLC with MeOH-H_2O (2:3) as eluent to give compound **1** (5.7 mg, t_R = 22 min) from fraction D, and rosmarinic acid (compound **11**, 20 mg, t_R = 18 min) and rosmarinic acid methyl ester (compound **12**, 7 mg, t_R = 41 min) from fraction E, respectively. Fraction C (443 mg) was

previously submitted to partition between *n*-BuOH and H_2O yielding a *n*-BuOH residue (67.8 mg) which was subsequently subjected to RP-HPLC with MeOH-H_2O (1:1) as eluent to yield pinocembrin 7-rutinoside (compound **13**, 2 mg, t_R = 15 min). Fraction F (330 mg) was purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to give rosmarinic acid (compound **11**, 10 mg, t_R = 9 min) and hesperetin (compound **3**, 5 mg, t_R = 24 min).
The structures of compounds **2-13** are showed in **Figures 4.3**, **4.4** and **4.5**

4.4 New isolated compound

2-*O*-Benzoyl-3-*O*-cinnamoyl tartaric acid (**1**)

Amorphous powder; $[a]_D^{25}$ -70 (*c* 0.2, MeOH); UV (MeOH) λ_{max}/nm (log ε) 213 (4.20), 225 (3.82), 309 sh (4.10); HR-ESI-MS *m/z*, calcd. for $C_{20}H_{16}O_8Na$ $[M+Na]^+$: 407.0743, found: 407.1691, 277.2327 $[M+Na-130]^+$, 131.3031 $[M+Na-130-146]^+$; ESI-MS *m/z*, 383 $[M-H]^-$; ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) data see **Table 4.1**.

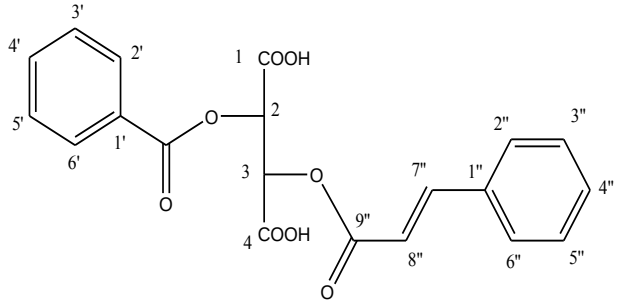


Figure 4.2: Structure of compound **1**

4.5 Structural elucidation

The chloroform-methanol and the methanol extracts of the aerial parts of *C. tomentosum* were subjected to Sephadex LH-20 column chromatography followed by reverse phase high performance liquid chromatography (RP-HPLC), to afford one new compound (**1**) (**Figure 4.1**) and twelve known compounds (**2-13**) (**Figure 4.3-4.5**).

Compound **1** was isolated as amorphous solid. Its molecular formula was determined as $C_{20}H_{16}O_8$ by HR-ESI-MS (*m/z* 407.1691 $[M+Na]^+$). Its HR-ESI-MS/MS spectrum showed two main fragments at *m/z* 277.2327 $[M+Na\text{-}130\ (C_9H_6O)]^+$ (95%) and 131.3031 $[M+Na\text{-}130\ (C_9H_6O)\text{-}146\ (C_9H_6O_2)]^+$ (28%) due to the loss of two asymmetric ester moieties. The ^1H and ^{13}C NMR spectra (**Table 4.1**) showed typical signals of a *trans*-double bond together with other five aromatic [δ_H 7.45 (3H, overlapped, H-3"/5" and H-4"), 7.50 (2H, t, *J* 7.5, H-3'/5'), 7.62 (1H, overlapped, H-4'), 7.65 (2H, overlapped, H-2"/6"), 8.17 (2H, t, dd, *J* 7.5, 1.5, H-2'/6')] and two hydroxymethines signals at δ_H 5.80 (1H, d, *J* 2.7) and 5.82 (1H, d, *J* 2.7). This information in conjuction with the remaining NMR signals and HR-ESI-MS/MS spectra, indicated the presence of a tartaric acid esterified with one benzoyl and one cinnamoyl residues. All the ^1H and ^{13}C NMR signals were assigned with the aid of 2D NMR spectra including 1D-TOCSY, DQF-COSY, HSQC, and HMBC spectra. The downfield shift of H-2 and H-3 (δ 5.80 and 5.82) and C-2 and C-3 (both 76.0 ppm) compared to tartaric acid confirmed that these positions were esterified (Soicke *et al*., 1988). The configuration of C-2 and C-3 remained undetermined. On the basis of all these evidences the structure of **1** was determined as 2-*O*-benzoyl-3-*O*-cinnamoyl tartaric acid. Asymmetric esters of tartaric acid are found rarely in nature, being isolated mostly from *Echinacea* genus (Soicke *et al*., 1988; Lu *et al*., 2012).

The following known compounds were identified by spectral analysis and comparison with published spectroscopic data: caffeic acid (**2**), caffeic acid methyl ester (**8**) (Saleem *et al.*, 2004) hesperitin (**3**) (Wagner *et al*., 1976), blumenol c glucoside (**4**) (Pabst *et al*.,1992), syringaresinol 4'-*O*-β-D-glucopiranoside (**5**) (Shahat *et al*., 2004), dihydrodehydroconiferyl alchol 9'-*O*-β-D-glucopyranoside (**6**) (Abe and Yamauchi, 1986), *p*-coumaric acid (**7**) (Ralph *et al*., 1994), caffeic acid ethyl ester (**9**) (Sugiura *et al*., 1989), clinopodic acid E (**10**) (Murata *et al.*, 2009), rosmarinic acid (**11**), rosmarinic acid methyl ester (**12**) (Eicher *et al.*, 1996) and pinocembrin 7-rutinoside (**13**) (Xu *et al*., 2011).

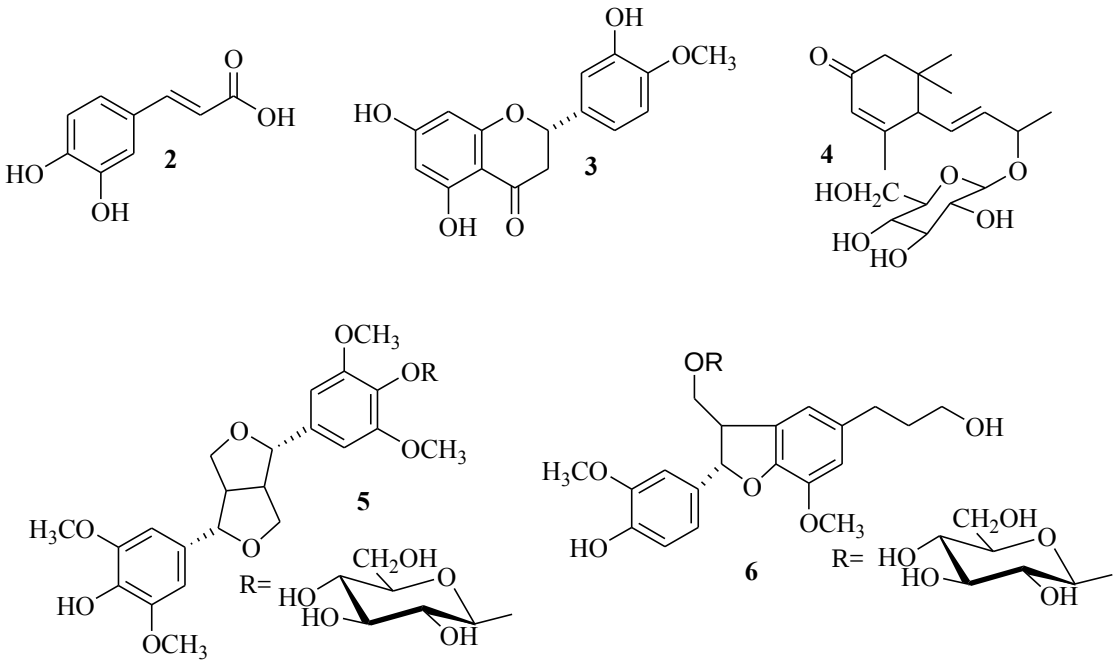


Figure 4.3: Structures of known compounds from *C. tomentosum*

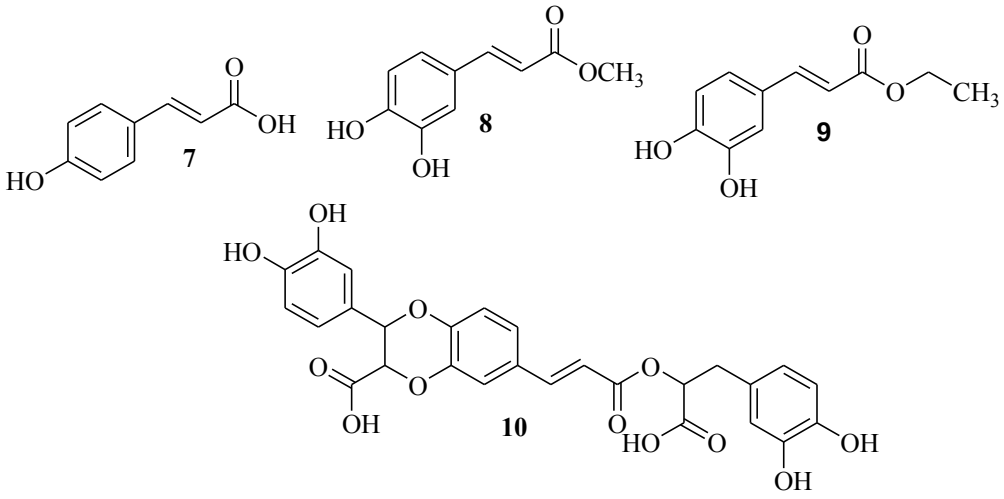


Figure 4.4: Structures of compounds **7** to **10** from *C. tomentosum*

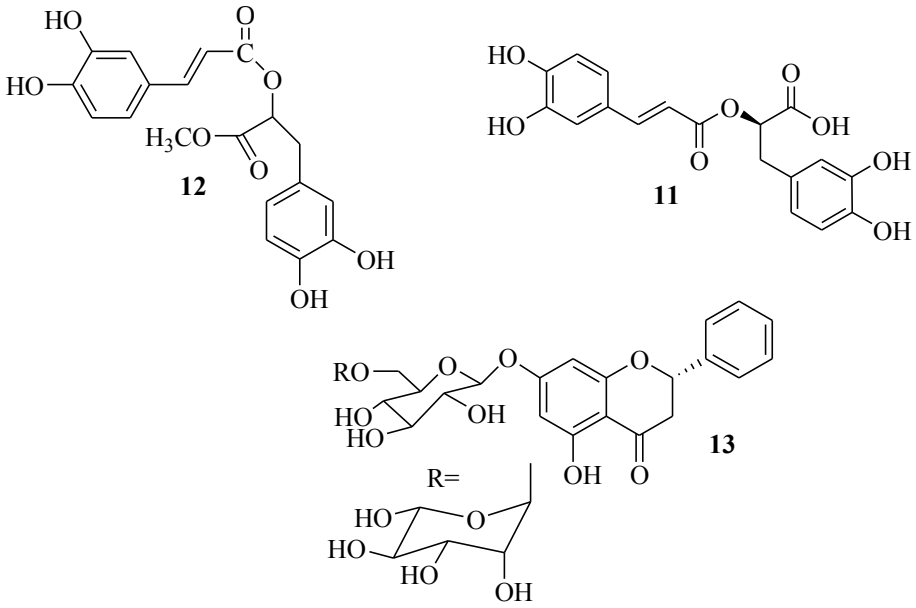


Figure 4.5: Structures of compounds **11-13** from *C. tomentosum*

Table 4.1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of compound **1** (CD_3OD)[a]

Position	δ_H (*J*/Hz)	δ_C
1/4		173.0
2	5.80 d (2.7)	76.0
3	5.82 d (2.7)	76.0
Benzoyl 1'		128.5
2'/6'	8.17 dd (7.5, 1.5)	133.6
3'/5'	7.50 t (7.5)	129.1
4'	7.62[o]	133.6
COO		167.2
Cinnamoyl 1'		134.3
2''/6''	7.65[o]	128.8
3''/5''	7.45[o]	130.0
4''	7.45[o]	130.0
7''	7.80 d (16.0)	146.2
8''	6.71 d (16.0)	118.4
9''COO		167.2

[a] chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments
[o] overlapped signals

CHAPTER 5

Phytochemical study of *Euphorbia laurifolia* Juss. ex Lam.

Chapter 5

4.1 Introduction: *Euphorbia* genus

Euphorbia genus belongs to Euphorbiaceae family and contains at least 2000 species. It's considered as one of the most diverse group of flowering plants on the earth. The species of this genus are known for their abundant content of diterpenes, which have wide biological activity, since they demonstrated antitumoral, antiviral and antifungal properties (Avila *et al*., 2010). Diterpenes, as main secondary metabolites of these plants, have provided many lead compounds for drug development due to their structural diversity including polycyclic and macrocyclic skeletons and various aliphatic and aromatic ester groups (Faiella *et al*., 2012).

All the species of *Euphorbia* genus produce white latex that they exude when cut, and this sap is demonstrated to be often toxic. Even if there are many herbaceous plants, the most part of the species seems to be succulent; some of them appear very similar to cacti.

The flowers, male or female, are unisexual, putted together into a cluster known as "cyathium", that is a particular characteristic of the genus. The cyathium features show modifications within the different groups of plants. The fruit is a capsule, about 8-12 mm, that normally split open when ripe and potentially contains three seeds (Motta, 1960).

Many species of the genus have been used in folk medicine as anti-inflammatory, analgesic, antipyretic agents, for treatment of cancer, diarrhea, warts and skin diseases. (Mwine and Van Damme, 2011; Avila *et al*., 2010).

Euphorbia laurifolia Juss. ex Lam. (**Figure 5.1**) is wildly distributed in Colombia, Venezuela, Ecuador, Peru and Bolivia. In Ecuador is easily found all over the provinces of the Andes, from 1500 up to 3000 meters above sea level. It corresponds to a glabrous shrub, 2-6 meters high, with stout branches, apically foliate and axillary cymes. The upper peduncles have 3 heads; leaves crowed, shortly petioled, oblanceolated- oblong, entire, 1-1.5 dm long, 2-3 cm wide, at least the lower more or less spreading-deflexed. With green cyathia, 5 mm long and wide, glands fleshy, 1-1.5 mm long, 3.5 wide, hirsute without,

otherwise glabrous. The stamens correspond to 25-30 filaments, 4 mm long, yellow anthers, 1-1.5 mm long and lacerate-hirsute bracts, 2-5 mm long (Macbride, 1951).

The wood is use as raw material for homebuilding, the milky sap or latex is suggested to have a protective and defensive role in helping heal wounds and in deterring potential plant-eaters. There is not too much information reported about the uses of this species, but leaves and latex seem to be used as infusion for cough (Tene *et al*., 2007). The plant is also used as living fences and as ornamental in some parks of Quito.

Figure 5.1 *Euphorbia laurifolia* Juss. ex

4.2 Plant material

Aerial parts of *E. laurifolia* were collected in Tumbaco, Ecuador in September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 4498 Euphorbia laurifolia/1) was deposited at Herbarium Horti Botanici Pisani, Pisa, Italy.

4.3 Extraction and isolation

The dried powdered leaves of *E. laurifolia* (527 g) were successively extracted for 48 h with *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1) and MeOH, by exhaustive maceration (3 x 2L), to give 15.2, 14.6, 2.7 and 20.2 g of the respective residues. The *n*-hexane extract (15.2 g) was partitioned between *n*-hexane and MeOH-H_2O (3:2), to afford a polar fraction (0.473 g) that was chromatographed over silica gel column using Biotage® Isolera™ Spektra flash purification system, eluting with *n*-hexane-$CHCl_3$ (1:1) follow by increasing concentrations of $CHCl_3$ in *n*-hexane (between 50 % and 100%), then by increasing concentrations of MeOH in $CHCl_3$ (between 3% and 10%). Fractions of 12 ml were collected, analyzed by TLC (silica gel plates, in mixture of *n*-hexane-$CHCl_3$ 1:1, $CHCl_3$ and mixtures of $CHCl_3$-MeOH 98:2, 95:5, 9:1), and grouped into 3 major fractions (A-C). Fraction B (295.6 mg) was purified by HPCPC (High Performance Centrifugal Partition Chromatography) with *n*-hexane-AcOEt-MeOh:H_2O (8:5:5:2), in which the stationary phase consisted of the upper phase (descending mode, flow rate of stationary phase 10 ml/min and of mobile phase 3 ml/min); 155 tubes were collected that give three major fractions (A-C) grouped by TLC. Fraction B (20.2 mg), was purified by RP-HPLC with MeOH-H_2O (7.5:2.5) as eluent to afford a new diterpene 7β,8α,15β-triacetoxy-3β-(2-methylpropanoyloxy)-4α,9αH,11αH-lathyra-5*E*,12*E*-dien-14-one (compound **1**, 0.5 mg, t_R = 10 min) and latazienone (compound **2**, 0.7 mg, t_R = 8 min).

Part of the $CHCl_3$ extract (5 g) was chromatographed over silica gel column using Biotage® Isolera™ Spektra flash purification system, eluting with *n*-hexane-$CHCl_3$ (1:1) followed by increasing concentrations of $CHCl_3$ in *n*-hexane (between 50 % and 100%), then by increasing concentrations of MeOH in $CHCl_3$ (between 5% and 20%). Fractions of 27 ml were collected, analyzed by TLC (silica gel plates, in mixture of *n*-hexane-$CHCl_3$ 7:3, 8:2, $CHCl_3$ and mixtures of $CHCl_3$-MeOH 99:1, 98:2, 97:3, 95:5, 9:1, 85:15), then grouped into 10 major fractions (A-J). Fraction D (186 mg) was subjected to RP-HPLC with MeOH-H_2O (5.5:4.5) as eluent to give isofraxidin (compound **3**, 18 mg, t_R = 11 min). Fraction E (486 mg) was purified by RP-HPLC with MeOH-H_2O (8.5:1.5) as eluent to give another new diterpene, 2α-hydroxy-*ent*-abieta-8,13-dien-12,16-olide (compound **4**, 1.5 mg, t_R = 7 min). Fraction F (180.5 mg) was purified over RP-HPLC with MeOH-H_2O (7:3) as eluent yield *ent*-16α,17-dihydroxykauran-3-one (compound **5**, 2.2 mg, t_R = 12 min). Fraction G

(485.6 mg) was subjected to RP-HPLC with MeOH-H_2O (6.5:3.5) as eluent to give vomifoliol (compound **6**, 3.1 mg, t_R = 6 min) and another pure known compound *ent*-16α, 17-dihydroxyatisan-3-one (compound **7**, 1.3 mg, t_R = 17 min). Fraction H (220.9 mg) was purified over RP-HPLC with MeOH-H_2O (2:3) as eluent to give *p*-hydroxy phenylethyl alcohol (compound **8**, 3.6 mg, t_R = 12 min) and a new abietanolide 6,9,13-trihydroxy-megastigman-7-en-3-one (compound **9**, 2.5 mg, t_R = 24 min). Fraction I (190.9 mg) was subjected to RP-HPLC with MeOH-H_2O (5.5:4.5) as eluent to yield another new diterpene 18-hydroxy-9αH,11αH-lathyra-4(15),5(6)-dien-1,14-dione-18-β-D-glucopyranoside (compound **10**, 7.8 mg, t_R = 13 min).

The $CHCl_3$-MeOH extract (2.7 g) was chromatographed over Sephadex LH-20 to give 5 major fractions (A-E) grouped by TLC. Fraction B (449 mg) was purified over RP-HPLC with MeOH-H_2O (1:1) as eluent to give corchoinoside c (compound **11**, 3.1 mg, t_R = 7 min) and compound **10** (7.4 mg, t_R = 16 min). Fraction C (475 mg) was subjected to RP-HPLC with MeOH-H_2O (1:4) as eluent to yield compound **8** (2.5 mg, t_R = 13 min) and scopoletin (compound **12**, 1.6 mg, t_R = 34 min).

The MeOH extract (20.1 g) of *E. laurifolia* was partitioned between *n*-BuOH and H_2O, to afford a *n*-BuOH residue (2.9 g). The *n*-BuOH fraction was submitted to Sephadex LH-20 using MeOH as eluent to obtain eight major fractions (A-H) grouped by TLC. Faction G results into a pure compound, quercetin 3-*O*-β-D-glucuronopyranoside (compound **13**, 35.5 mg). Fraction B (407.4 mg) was purified over RP-HPLC with MeOH:H_2O (4.5:5.5) as eluent to give compound **10** (1.6 mg, t_R = 13 min), corchoinoside c (compound **11**, 2.5 mg, t_R = 7 min), as well as the new compounds, 18-hydroxy-9αH,11αH-lathyra-4(15),5(6)-dien-1,14-dione-18-β-D-glucopyranoside (compound **14**, 1.2 mg, t_R = 60 min), and 20-deoxy-16-hydroxyingenol-3-β-D glucopyranoside (compound **15**, 1.1 mg, t_R = 23 min). Fraction D (364.6 mg) was purified by RP-HPLC with MeOH-H_2O (3:7) as eluent to yield chlorogenic acid (compound **16**, 1.4 mg, t_R = 15 min), caffeic acid (compound **17**, 1.8 mg, t_R = 11 min), 4-*O*-caffeoyl quinic acid (compound **18**, 2.1 mg, t_R = 25 min), 4-*O*-caffeoyl quinic acid methyl ester (compound **19**, 1.2 mg, t_R = 40 min) and scopoletin (compound **12**, 2.1 mg, t_R = 65 min).

Fraction F (452.2 mg) was subjected to RP-HPLC with MeOH-H_2O (2:3) as eluent to give kaempferol 3-*O*-β-D-glucuronopyranoside (compound **20**, 10 mg, t_R = 30 min).
The structures of the new compounds are shown in **Figure 5.2**. The structures of the other compounds are reported in **Figure 5.3**.

4.4 New isolated compounds

7β,8α,15β-triacetoxy-3β-(2-methylpropanoyloxy)-4α,9αH,11αH-lathyra-5*E*,12*E*-dien-14-one (**1**)
Pale yellow oil; ^{1}H and ^{13}C NMR data, see **Table 5.2**; HR ESIMS *m/z* 569.2344 $[M+Na]^+$ (calcd for $C_{30}H_{42}O_9$ 546.2829), 509.2666 $[M+Na-60]^+$, 449.3008 $[M+Na-60-60]^+$, 389.3351 $[M+Na-60-60-60]^+$.

2α-hydroxy-*ent*-abieta-8,13-dien-12,16-olide (**4**)
Amorphous powder; ^{1}H and ^{13}C NMR data, see **Table 5.3**; HR ESIMS *m/z* 339.2778 $[M+Na]^+$ (calcd for $C_{20}H_{28}O_3$ 316.2038), 317.2920 $[M+H]^+$.

6,9,13-trihydroxy-megastigman-7-en-3-one (**9**)
Amorphous powder; $[\alpha]_D^{25}$ +8 (*c* 0.125, MeOH); ^{1}H and ^{13}C NMR data, see **Table 5.3**; HR ESIMS *m/z* 507.3278 $[2M+Na]^+$ (calcd for $C_{13}H_{22}O_4$ 242.1518).

18-hydroxy-9αH,11αH-lathyra-4(15),5(6)-dien-1,14-dione-18-β-D-glucopyranoside (**10**)
Pale yellow oil; $[\alpha]_D^{25}$ +19 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 245 (3.90), 230 sh; ^{1}H and ^{13}C NMR data, see **Table 5.1**; ESI-MS *m/z* 477 $[M-H]^-$, 315 $[M-H-162]^-$; HR ESIMS *m/z* 501.2439 $[M+Na]^+$ (calcd for $C_{26}H_{38}O_8$ 478.2567), 347.3342 $[M-H-162]^-$, 329.3456 $[M-H-162-18]^-$.

18-hydroxy-9αH,11αH-lathyra-4(15),5(6)-dien-1,14-dione-18-β-D-glucopyranoside (**14**)

Pale yellow oil; ^{1}H and ^{13}C NMR data, see **Table 5.1**; ESI-MS *m/z* 477 $[M-H]^-$; HR ESIMS *m/z* 501.2439 $[M+Na]^+$ (calcd for $C_{26}H_{38}O_8$ 478.2567).

20-deoxy-16-hydroxyingenol-3-β-D glucopyranoside (**15**)
pale yellow oil; ^{1}H and ^{13}C NMR data, see **Table 5.2**; HR ESIMS *m/z* 509.2432 [M-H]- (calcd for $C_{26}H_{38}O_{10}$ 510.2465), 347.3342 $[M-H-162]^-$, 329.3456 [M-H-162-18], 533.2091 $[M+Na]^+$, 353.3166 $[M+Na-180]^+$.

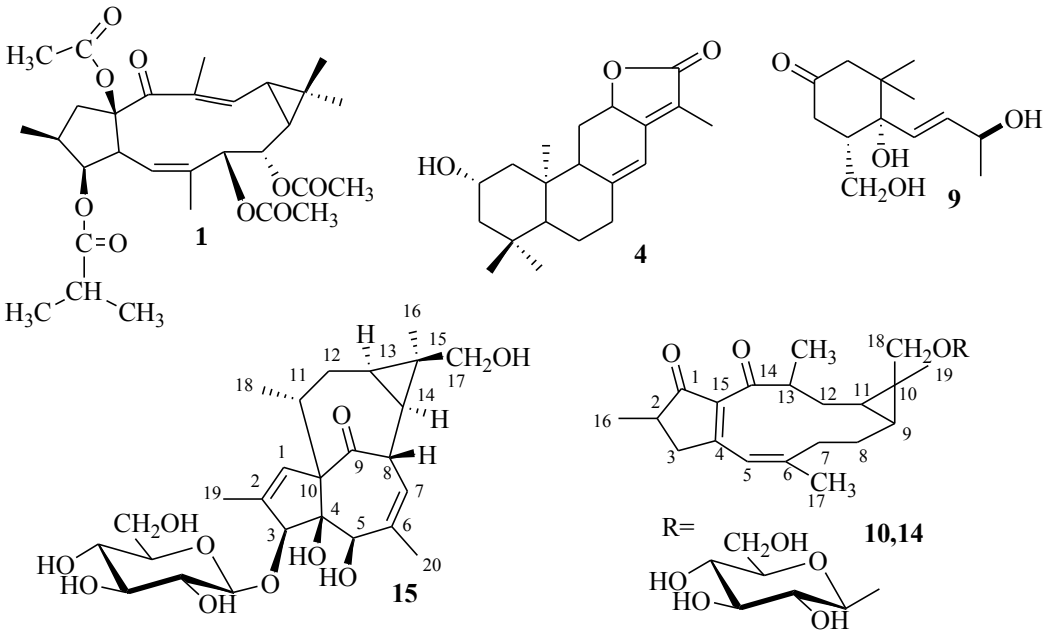


Figure 5.2: New isolated compounds from *E. laurifolia*

4.5 Structural elucidation and biological activity

The structures of all the following known compounds were determined by spectral analysis and comparison of data with those reported in the literature: latazienone (**2**) (Rondon *et al.*, 2005), isofraxidin (**3**) (Borris *et al*., 1980), *ent*-16α,17-dihydroxykauran-3-one (**5**) (Ding and Jia, 1991), vomifoliol (**6**) and corchoinoside c (**11**) (Calis *et al*., 2002), *ent*-16α, 17-dihydroxyatisan-3-one (**7**) (Lal *et al*., 1990), *p*-hydroxy phenylethyl alcohol (**8**), scopoletin (**12**) (Tsukamoto *et al*., 1984), quercetin 3-*O* -β-D-glucuronopyranoside (**13**) (Parejo *et al.*,

2004), chlorogenic acid (**16**), caffeic acid (**17**) (Saleem *et al*., 2004), 4-*O*-caffeoyl quinic acid (**18**), 4-*O*-caffeoyl quinic acid methyl ester (**19**) (Zhu *et al.*, 2005) and kaempferol 3-*O* -β-D-glucuronopyranoside (**20**) (Merfort and Wendisch, 1998).

The ^{13}C NMR spectrum of **15** showed 26 carbon atoms which can be recognized as four methyls, three methylenes, thirteen methynes, and six quaternary carbons. Moreover, the analysis of ^{13}C NMR chemical shifts showed the presence of a carbonyl, eleven oxygen-bearing carbon atoms and four sp^2 carbons. The molecular formula $C_{26}H_{38}O_{10}$ was established for **15** from ^{13}C NMR data and the HRESIMS spectrum (*m/z* 509.2432 $[M-H]^-$). The analysis of fragmentation pattern in the HRESIMS/MS experiment showed peaks at *m/z* 347.3342 $[M-H-162]^-$ and 329.3456 $[M-H-162-18]^-$ suggesting the presence of one hexose unit. The ^1H NMR aglycone portion spectrum of **15** revealed the presence of two olefinic protons at δ 5.70 (1H, br d, J = 4.0 Hz) and 5.88 (1H, s), two methyl groups bearing a double bond at δ 1.77 (3H, s) and 1.87 (3H, s), two hydroxymethynes at δ 3.34 and 3.31, as overlapped signal, and 4.50 (1H, s), and one hydroxymethyne at δ 3.70 and 3.71 (each 1H, d, *J* = 11.0 Hz). Additionally, two methynes at δ 0.84 (1H, br t, *J* = 8.5, 4.2 Hz) and 0.95 (overlapped signal) attributable to a cyclopropane ring were evident. The DQF-COSY and 1D-TOCSY spectra of **15** showed one main spin system from Me-18 to H-7. The interpretation of HSQC results permit to associate all the proton chemical shifts to those of the respective carbon resonances; then, the study of HMBC cross peaks was used to link the previous obtained spin system to the quaternary carbons, indicating that **15** was an ingenol derivative (Marco *et al.*, 1997) The chemical shifts of H-3 showed correlations with C-1, C-2, C-4, C-5, and C-10; H-7 with C-8, C-9 and Me-20; H-11 with C-9, C-10, C-13, and Me-18. Correlations of Me-20 with C-5, C-6, and C-9 and Me-16 with C-13, C-15, and C-17 led to establish the remaining structural connectivities. The position of the sugar moiety was ascertained to be at C-3 from the HMBC cross peak between $H\text{-}1_{glc}$ (δ 4.52) and C-3 (90.0 ppm). The relative stereochemistry of the stereogenic centers was partially determined from the cross peaks observed in the ROESY spectrum. Key correlations peaks were pointed out between H-8 and H-11, and H_2-17; H-3 and H-5; H-11 and H_2-17 and were in agreement with the relative stereochemistry reported in the literature for similar compound (Marco *et al.*, 1997; Bakhsh Baloch *et al.*, 2009). The configuration of the

glucopyranosyl moiety was determined to be D by hydrolysis, trimethylsilylation, and GC analysis. Consequently, the obtained data led to establish compound **15** as 20-deoxy-16-hydroxyingenol-3-β-D glucopyranoside.

Compounds **10** and **14** were obtained as yellow oils. Their HRESIMS exhibited a molecular ion peak at *m/z* 501.2439 $[M+Na]^+$ for **10** and 501.2437 $[M+Na]^+$ for **14**, respectively, which agreed with the molecular formula $C_{26}H_{38}O_8$, showing that they were two isomers. Another product ion detected in their HRESIMS spectra at *m/z* 317.2104 $[M+H-162]^+$ and 317.2101 $[M+H-162]^+$ was strongly suggestive of the presence of one hexose unit. The ^{1}H NMR, ^{13}C NMR (**Table 5.1**), and ^{13}C DEPT spectra of **10** indicated, besides signals attributable to one sugar moiety, the presence of two ketone groups, two double bonds (one trisubstituted and one tetrasubstituted), and 14 sp^3 carbons, including four methyls, five methylenes, four methynes, and one quaternary carbon. These observations were confirmed by the presence in the ^{1}H NMR spectrum of signals at δ 5.71 (1H, s) and δ 1.68 (3H, s); characteristic signals at δ 0.56 (1H, br t, *J* = 9.0 Hz) and 0.77 (1H, ddd, J = 13.0, 8.0, 3.0 Hz) suggested a cyclopropane ring, present in many types of diterpenes from Euphorbiaceae genus (Liao *et al.*, 2005). DQF-COSY, 1D-TOCSY and HSQC experiments of **10** allowed to establish the following connectivities: H-2—H-3 for ring A, H-7—H-20 for ring B, which led to recognize the structural moieties of a lathyrane diterpene (Rondon *et al*., 2005). Analysis of the chemical shifts, signal multiplicities, and absolute values of the coupling constants in the ^{1}H NMR spectrum, as well as ^{13}C NMR data, indicated the presence of one glucopyranosyl moiety with a β-configuration at the anomeric carbon. The configuration of the glucopyranosyl moiety was determined as reported for **15**. The chemical shift assignments of the carbon atoms of **10** were established from the HSQC and HMBC spectra. Key HMBC correlations between H_2-3—C-1, H_2-3—C-4, H_2-3—C-15; H_2-7—C-5, H_2-7—C-9, H_2-7—C-11, H_2-7—C-17; H-9—C-7, H-9—C-10, H-9—C-18; H-11—C-9, H-11—C-13; Me-16—C-1, Me-16—C-3; Me-17—C-4, Me-17—C-5, Me-17—C-7; H_2-18—C-1_{glc}; Me-20—C-11, Me-20—C-12, Me-20—C-14, were observed and allowed to locate the keto groups at C-1 and C-14, the conjugated double bonds at C-4/C-15 and C-5/C-6, and the glucopyranosyl moiety at C-18. The NMR

spectra of **14** were almost superimposable with those of **10**; differences were observed for the chemical shifts of C-2 (δ 2.62 and 41.0 in **10** *versus* δ 2.58 and 41.0 in **14**), C-3 (δ 2.97 and 2.53 and 37.2 in **10** *versus* δ 2.30 and 3.19 and 37.2 in **14**), and C-16 (δ 1.22 and 16.6 in **10** *versus* δ 1.29 and 16.5 in **14**). Therefore, it was possible to hypothesize that the stereochemistry at C-2 of A-ring in the two compounds was the point of difference. Thus, compounds **10** and **14** were elucidated as 18-hydroxy-9αH,11αH-lathyra-4(15),5(6)-dien-1,14-dione-18-β-D-glucopyranoside. The absolute configuration of both compounds is still in progress.

HRESIMS spectrum identified compound **1** as $C_{30}H_{42}O_9$ with molecular ion peak at *m/z* 569.2344 $[M+Na]^+$. The analysis of MS spectrum and ^{13}C NMR data showed ten degrees of unsaturation, seven of which attributable to four ester functionalities, two double bonds and one carbonyl group. Analysis of NMR spectral data evidenced that **1** was a tricyclic lathyrane diterpene. The study of NMR data for the diterpenoid core matched those for latazienone (Rondon *et al.,* 2005) being the esterification substitution the point of difference. Compound **1** differed from latazienone only in the replacement of the benzoyl group at C-7 with an acetyl group. Therefore, **1** was elucidated as 7β,8α,15β-triacetoxy-3β-(2-methylpropanoyloxy)-4α,9αH,11αH-lathyra-5*E*,12*E*-dien-14-one.

The molecular weight determined for compound **4** was 316 (HRESIMS at *m/z* 317.2920 $[M+H]^+$, $C_{20}H_{28}O_3$). The ^1H NMR spectrum of **4** revealed the presence of four methyls, one of which bearing a double bond, one olefinic proton, and two hydroxymethine. The ^{13}C NMR spectrum displayed twenty signals including four sp^2 carbons and one ester functionality; being the unsaturation degree seven, compound **4** was found to be tetracyclic. Analysis of DQF-COSY and HSQC experiments showed the fragments H-1—H-3, H-5—H-7, and H-9—H-12. The inspection of all NMR data revealed signals attributable to a helioscopinolide diterpene. The position of hydroxyl group at C-2 was based on the chemical shifts of C-1, C-2, and C-3 and the observed HMBC correlations between H-1—C-3, H-2—C-10, H-3—C-2; the relative stereochemistry of OH-2 was established as 2α from the H-2 chemical shift (δ 3.84) and coupling constant of H-1 (δ 2.23, br d, J = 14.0

Hz, 1.08, dd, *J* = 14.0, 4.5 Hz) and H-2 (δ 3.84, 1H, br dd, *J* = 4.5, 3.5 Hz). Thus, **4** was identified as 2α-hydroxy-*ent*-abieta-8,13-dien-12,16-olide.

Compound **9** was isolated as an amorphous powder and showed in the HRESIMS a peak at *m/z* 507.3278, which supported by NMR data, suggested the molecular formula to be $C_{13}H_{22}O_4$. The ^{1}H and ^{13}C NMR spectra (**Table 5.3**) indicated that compound **9** was a megastigmane derivative with a carbonyl group at C-3, hydroxyl functional group at C-6 and a disubstituted double bond. A resonance for a hydroxymethylene was also observed in the ^{1}H NMR (δ_H 3.85, dd, *J* = 10.5, 5.0 Hz, and δ_H 3.61, dd, *J* = 10.5, 3.0 Hz) and in the ^{13}C NMR (δ_C 64.0). The elucidation of the whole skeleton from the above subunits was achieved on the basis of HSQC and HMBC correlations, which also allowed the assignment of all the resonance in the ^{13}C NMR spectrum of the pertinent carbons. According to HMBC spectrum the hydroxymethylene protons showed a long range correlation with C-4 (δ_C 41.5), C-5 (δ_C 43.6), and C-6 (δ_C 78.1), while the double bond proton at δ 5.80 (H-7) exhibited a long range correlation with C-6 and C-9 signals, indicating that hydromethylene function was located at C-5, while the double bond at carbons C-7 and C-8 (De Marino *et al*., 2004). The relative stereochemistry determination of compound **9** is still under progress but basing on literature data (De Marino *et al*., 2004) it can be supposed to be as reported in **Figure 5.2**. These findings suggested that the structure of **9** was 6,9,13-trihydroxy-megastigman-7-en-3-one.

The antiproliferative activity of compound **10** was preliminary assayed against human prostate cancer (PC-3) and human breast adenocarcinoma (MCF7) cell lines, showing an IC_{50} value of 25 μM at 24 and 48 h. In order to identify compound **10** molecular target a DARTS method was performed. The LC-MS analyses following in-gel trypsin digestions led to the identification of the proteins reported below (**Table 5.4**). Clathrin heavy chain 1, that is involved in the internalization of exogenous compounds, appeared both in PC3 and MCF7 cell lines according with previous reports of lathyrane diterpenes (Reis *et al*., 2013). Moreover **10** seems to influence the synthesis and translocation of ATP and the acylCoA metabolism. More investigations will be carried out to confirm these preliminary results.

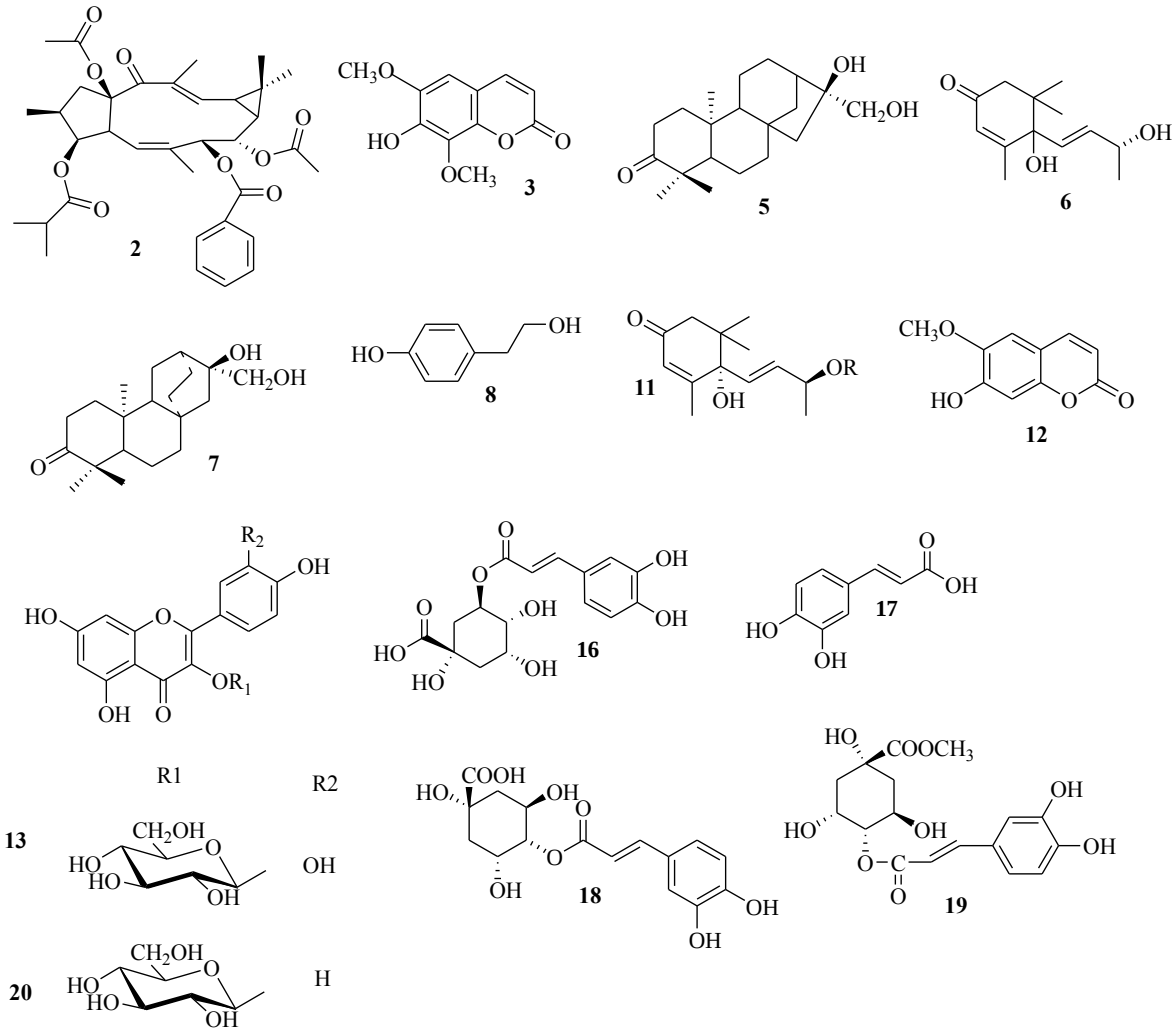


Figure 5.3: Structures of known compounds isolated from *E. laurifolia*

Table 5.1. ^{1}H and ^{13}C NMR Data of Compounds **10** and **14** (Methanol-d_4, 600 MHz)[a]

	10		**14**	
position	δ_H	δ_C	δ_H	δ_C
1		213.4		213.2
2	2.62 m	40.9	2.58 m	41.0
3a	2.97 dd (14.0, 3.5)	37.2	3.19[b]	37.2
3b	2.53 dd (14.0, 2.0)		2.30 dd (18.0, 16.0)	
4		169.3		169.5
5	5.71 s	116.6	5.71 s	116.5
6		145.9		145.4
7a	2.37 m	39.4	2.36 m	39.4
7b	2.25 br t (12.0)		2.22 br t (12.0)	
8a	1.89[b]	23.6	1.88[b]	23.5
8b	1.18 m		1.18 m	
9	0.56 br t (9.0)	26.2	0.55 br t (9.2)	26.1
10		21.7		21.7
11	0.77 ddd (13.0, 8.0, 3.0)	22.7	0.76 ddd (13.5, 9.0, 5.0)	21.4
12a	1.89[b]	27.5	1.88[b]	27.4
12b	1.40 ddd (15.0, 8.0, 3.0)		1.40 ddd (15.0, 7.5, 3.0)	
13	3.32[b]	44.6	3.33[b]	44.2
14		211.4		211.4
15		142.0		142.0
16	1.22 d (6.5)	16.6	1.29 d (6.5)	16.5
17	1.68 s	20.7	1.66 s	20.6
18a	3.59 d (11.0)	80.4	3.58 d (11.0)	80.4
18b	3.40 d (11.0)		3.39 d (11.0)	
19	1.02 s	11.5	1.02 s	11.0
20	1.07 d (6.5)	17.3	1.08 d (6.5)	17.9
Glc 1'	4.31 d (7.5)	103.6	4.31 d (7.5)	103.2
2'	3.22 dd (9.0, 7.5)	75.0	3.21 dd (9.0, 7.5)	74.7
3'	3.29 t (9.0)	77.6	3.27 t (9.0)	77.7
4'	3.34 t (9.0)	71.3	3.34 t (9.0)	71.4
5'	3.37 m	78.0	3.34 m	78.0
6'a	3.88 dd (12.0, 3.5)	62.5	3.88 dd (12.0, 3.0)	62.3
6'b	3.71 dd (12.0, 5.0)		3.72 dd (12.0, 4.5)	

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments. [b] overlapped signal.

Table 5.2. ^{1}H and ^{13}C NMR Data of Compounds **1** and **15** (Methanol-d_4, 600 MHz)

	15		**1**	
position	δ_H	δ_C	δ_H	δ_C
1a	5.88 s	131.0	2.81 dd (15.0, 6.2)	42.2
1b			2.19 dd (15.0, 7.8)	
2		140.0	2.32 m	39.0
3	4.50 s	90.0	4.85[b]	83.0
4		87.6	2.84 dd (6.2, 3.4)	47.8
5	3.34[b]	76.4	5.85 d (10.7)	124.6
6		140.6		140.8
7	5.70 br d (4.0)	123.6	4.88[b]	77.8
8a	4.38 br d (11.0)	44.4	5.29 br t (10.5)	73.8
8b			1.18 m	
9		211.0	1.43 br t (8.5)	34.6
10		73.6		26.4
11	2.44 m	40.6	1.80 dd (12.0, 8.5)	30.7
12			6.66 d (12.0)	145.6
12_a	2.40 m	31.3		
12_b	1.86[b]			
13	0.84 br t (8.5, 4.2)	24.6		134.3
14	0.95[b]	24.0		195.8
15		31.0		96.0
16	1.14 s	24.6	1.05 d (6.5)	18.4
17			1.61 s	18.2
17_a	3.71 d (11.0)	62.8		
17_b	3.70 d (11.0)			
18	0.95 d (6.5)	17.0		
18a			1.24 s	28.5
19			1.11 s	17.0
20			1.83 s	12.3
Glc 1'	1.87 s	15.6		
2'	1.77 s	22.3		
3'	4.52 d (7.5)	104.8		
4'	3.20 dd (9.5, 7.5)	75.0		
5'	3.38 t (9.5)	78.0		
6'a	3.36 t (9.5)	71.0		
6'b	3.40 m	78.0		
7-$\underline{CH_3}CO$			2.00 s	20.7
$CH_3\underline{CO}$				171.3
8-$\underline{CH_3}CO$			2.12 s	21.7
$CH_3\underline{CO}$				171.6
15-$\underline{CH_3}CO$			2.12 s	21.7
$CH_3\underline{CO}$				171.6
Isobut 1				177.3
2			2.35 sept (7.0)	35.6
3/4			1.26 d (6.5)	19.5

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.
[b] overlapped signal.

Table 5.3. ^{1}H and ^{13}C NMR Data of Compound **4** and **9** (Methanol-d_4, 600 MHz)

	4		**9**	
position	δ_H	δ_C	δ_H	δ_C
1a	2.23 br d (14.0)	49.6		43.6
1b	1.08 dd (14.0, 4.5)			
2a	3.84 br dd (4.5, 3.5)	66.8	2.96 d (13.5)	53.0
2b			1.86 d (13.5)	
3a	1.79 dd (12.5, 5.0)	51.9		215.2
3b	1.21 dd (12.5, 3.5)			
4a		35.0	2.80 t (13.5)	41.5
4b			2.29 dq (13.5, 6.7, 4.0, 2.0)	
5	1.26 dd (12.0, 3.0)	56.0	2.21 m	43.6
6a	1.86 m	27.0		78.1
6b	1.57 m			
7a	2.58 br d (13.0)	37.0	5.80 d (15.5)	132.0
7b	2.29 m			
8		152.2	5.98 dd (15.5, 6.2)	137.6
9	2.39 d (8.5)	53.0	4.37 q (12.6, 11.5, 6.0, 4.7)	69.0
10		42.0	1.29 d (6.0)	24.4
11a	2.63 dd (13.0, 7.0)	28.8	0.92 s	24.0
11b	1.52 m			
12	4.99[b]	77.2	0.92 s	24.0
13a		157.7	3.85 dd (11.0, 5.0)	64.0
13b			3.61 dd (11.0, 2.5)	
14	6.49 s	113.4		
15		116.8		
16		176.0		
17	0.94 s	23.3		
18	1.00 s	34.0		
19	1.03 s	34.7		
20	1.70 s	8.9		

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.
[b] overlapped signal.

Table 5.4. Proteins identified in PC3 and MCF7 cell lines by DARTS method

PC3	**MCF7**
Heat shock cognate 71kDa protein	ADP/ATP translocase 2
ATP synthase subunit alpha	Clathrin heavy chain 1
Dihydrolipoyl dehydrogenase	3-hydroxyacyl-CoA dehydratase 3
Protein disulfide-isomerase A4	
Clathrin heavy chain 1	
ATP synthase subunit β	
Threonine-tRNA ligase	
Very long-chain specific acyl-CoA dehydrogenase	
Myoferlin	

CHAPTER 6

Phytochemical study of *Bidens humilis* Kunth syn. Bidens triplinervia Kunth.

Chapter 6

6.1 Introduction: *Bidens* genus

The genus *Bidens* L., Asteraceae family, comprises about 240 species of annual or perennial herbs, widely distributed in tropical and subtropical countries, especially in America. The chemical study of many of these species has been carried out in order to obtain chemotaxonomic information to contribute to the classification of the Asteraceae, on the basis of the different compounds found, as acetylenes, sesquiterpenes lactones and flavonoids (Silva *et al.*, 2011). Plants of the genus are used in many indigenous systems of medicines as anti-inflammatory, antiallergic, antibacterial, antidiabetic, antimalarial, antiviral, antihypertensive, and antioxidant remedies (Bairwa *et al*., 2010). *Bidens* species are mainly rich in addition of the compounds mentioned before in phenol glycosides (De Tommasi *et al*., 1998; Wang *et al*., 2003).

Bidens humilis Kunth syn. *Bidens triplinervia* Kunth (**Figure 6.1**) is a plant used in Ecuadorian folk medicine for the treatment of headache, fever and liver diseases, while the flower infusion against gastric and abdominal pain. Moreover, the indigenous Salasacas used the yellow flowers to dye wool and other clothes. Nevertheless, no phytochemical study on this species was reported in the literature. For this species, an antioxidant-oriented approach was carried out on the aerial part plant extracts.

Figure 6.1: *Bidens humilis*

6.2 Plant material

Aerial parts of *Bidens humilis* Kunth were collected in Tumbaco, Ecuador, on September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 9237 Bidens humilis/1) was deposited at Herbarium Horti Botanici Pisani, Nuove Acquisizioni, Pisa, Italy.

6.3 Extraction and isolation

The dried aerial parts of *B. humilis* (610 g) were extracted for 48 h with solvents of increasing polarity, *n*-hexane, $CHCl_3$ and MeOH by exhaustive maceration (2 L), to give 5.7, 16.0, 26.8 g of the respective residues. The MeOH extract was partitioned between *n*-BuOH and H_2O, to afford a *n*-BuOH residue (4.0 g). Part of the *n*-BuOH extract (2.5 g) was submitted to a Sephadex LH-20 column (3 x 100 cm, flow rate 1.0 mL/min) using MeOH as eluent and collecting 56 fractions of 10 mL that were grouped by TLC into six major fractions (A-F). Fractions B (230 mg, 230-290 mL), C (122 mg, 300-330 mL), and E (93.6 mg, 390-480 mL) were subjected to RP-HPLC with MeOH–H_2O (3:7) as eluent to give pure compounds **5** (2.4 mg, t_R =33 min), **10** (3.6 mg, t_R = 46 min), and **6** (3.7 mg, t_R = 100 min) from fraction B; compounds **3** (5.1 mg, t_R = 23 min), **1** (5.3 mg, t_R = 32 min), and **9** (2.0 mg, t_R = 53 min) from fraction C; compound **11** (2.2 mg, t_R = 16 min) from fraction E, respectively. Fraction D (185 mg, 340-380 mL) was purified by RP-HPLC with MeOH–H_2O (2:3) as eluent to give pure compounds **2** (1.6 mg, t_R = 32 min), **4** (2.5 mg, t_R = 40 min), and **8** (4.0 mg, t_R = 90 min). Fraction F (490-560 mL) yielded pure compound **7** (14.5 mg).

The structures of compounds **1**-**11** are showed in **Figures 6.2**

6.4 New isolated compounds

(2S)-Isookanin 7-*O*-α-L-arabinopyranoside (**1**)

Yellow amorphous powder; $[\alpha]_D^{25}$ -22 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 281 (4.31), 327 (3.87); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) – 5020 (274 nm), + 4380 (306 nm); ^{1}H and ^{13}C NMR data, see **Table 6.1**; ESI MS *m/z* 419 $[M-H]^-$, 287 $[(M- 132)-H]^-$; HR ESIMS $[M-H]^-$ 419.0984 (calcd for $C_{20}H_{19}O_{10}$ 419.0978), 287.0602 $[(M-132)-H]^-$, 151.0058 $[C_7H_4O_4-H]^-$, 135.0049 $[C_7H_4O_3-H]^-$.

(2S)-Isookanin 7-*O*-(2"-acetyl)-α-L-arabinopyranoside (**2**)

Yellow amorphous powder; $[\alpha]_D^{25}$ -29 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε): 283 (4.12), 330 (3.98); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) – 6400 (275 nm), + 4560 (307 nm); ^{1}H and ^{13}C NMR data, see **Table 6.1**; ESI MS *m/z* 461 $[M-H]^-$; HR ESIMS *m/z* 461.1090 $[M-H]^-$ (calcd for $C_{22}H_{22}O_{11}$ 461.1084), 401.1067 $[(M-60)-H]^-$, 287.0865 $[(M-174)-H]^-$, 151.0043 $[C_7H_4O_4-H]^-$ and 135.0041 $[C_7H_4O_3-H]^-$.

Luteolin 7-*O*-β-D-glucopyranosyl-(1→6*)*-β-D-galactopyranoside (**6**)

Yellow amorphous powder; $[\alpha]_D^{25}$ -45 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 270 (4.33), 335 (3.96); ^{1}H and ^{13}C NMR data, see **Table 6.1**; ESI MS *m/z* 609 $[M-H]^-$, 447 $[(M-162)-H]^-$, 285 $[(M-162-162)-H]^-$, 633 $[M+Na]^+$; HR ESIMS *m/z* 609.1461 $[M-H]^-$ (calcd for $C_{27}H_{29}O_{16}$ 609.1458).

6.5 Structural elucidation and biological activity, antioxidant activity of extracts, fractions and pure compounds

The aerial parts of *B. humilis* were sequentially extracted with solvent of increasing polarity giving *n*-hexane, chloroform, and methanol residues. The methanol extract was partitioned between *n*-BuOH and water, yielding a *n*-BuOH fraction. All extracts were subjected to the 2,2-Diphenyl-2-picrylhydrazyl (DPPH), β-carotene bleaching (BCB) and ferric reducing antioxidant power (FRAP) assays to screen their antioxidant activity. Total Phenolic Content (TPC) was also measured. A single assay cannot determine completely the

antioxidant activity of a phytocomplex, thus different approaches are needed to study its biological potential (Girones-Vilaplana *et al*., 2012). For this reason the antioxidant activity was tested by using three complementary systems. A new concept, Relative Antioxidant Capacity data Index (RACI) was applied integrating antioxidant capacity data determined by the different methods. TPC results were included in RACI calculation since phenolics can act with other mechanisms (not measurable with our tests) and can contribute significantly to phytocomplex health promoting value. Moreover it was recently proposed that results obtained by Folin-Ciocalteu procedure could also be interpretedas an alternative way to measure the total reducing capacity of extracts as the reagent reacts with any reducing substance (Fernandes *et al*., 2013) In this way RACI provided a more comprehensive assessment of the whole antioxidant potential. The *n*-BuOH fraction demonstrated to be the one containing the highest concentration of total phenolics (from 8 to 20 times higher than the other extracts) (**Table 6.1**). The high phenolic content of a phytocomplex is usually directly related to its antioxidant activity measured with DPPH and FRAP tests, while there are evidences that it is not the same with BCB test (Milella *et al*., 2014). This phenomenon can be explained by the affinity of the antioxidant complex for the lipids and thus the lipophilic nature of BCB test could be the determining factor (Milella *et al*., 2014; Von Gadow *et al*., 1997). Our results confirmed this evidence since the *n*-BuOH extract was more active as radical scavenger (DPPH) and as antioxidant (FRAP) than as antilipoperoxidative (BCB). RACI was calculated to examine obtained results and the *n*-BuOH extract was confirmed to be the most active (**Figure 6.3a**). The *n*-BuOH extract was subjected to Sephadex LH-20 column chromatography collecting six major fractions. Also in this case all fractions were evaluated for their TPC and antioxidant activities (**Table 6.1**). All fractions are rich in phenolics with a total content higher than 361.8 mg of gallic acid equivalents per g of extract (mgGAE/g) (fraction C) to 518.0 mgGAE/g for fraction F, with the exception of fraction A (90.1 mgGAE/g). Fraction E showed the highest DPPH and FRAP values [857.3 and 2208.8 mg of Trolox equivalent per g (mgTE/g) of extract respectively], while content. Fraction F showed the presence of one spot on TLC and was directly subjected to spectroscopic and spectrometric analyses. Fractions B-E were purified by RP-HPLC; totally eleven compounds the highest BCB

value was registred for fraction F. On the basis of RACI results (**Figure 6.3a**) and on the evidence that the most promising fractions should have at least one value higher than their relative extract, fraction A was excluded from further analysis. All selected fractions were investigated for their phytochemical (**1**-**11**) were characterized (**Figure 6.2**) of which three were new natural flavonoids (**1**, **2**, and **6**). Fraction E showed the highest DPPH and FRAP values [857.3 and 2208.8 mg of Trolox equivalent per g (mgTE/g) of extract respectively], while the highest BCB value was registred for fraction F. On the basis of RACI results (**Figure. 2a**) and on the evidence that the most promising fractions should have at least one value higher than their relative extract, fraction A was excluded from further analysis. All selected fractions were investigated for their phytochemical content. Fraction F showed the presence of one spot on TLC and was directly subjected to spectroscopic and spectrometric analyses. Fractions B-E were purified by RP-HPLC; totally eleven compounds (**1**-**11**) were characterized (**Figure. 1**) of which three were new natural flavonoids (**1**, **2**, and **6**).

A molecular formula of $C_{20}H_{20}O_{10}$ was assigned to compound **1** on the basis of its HR ESIMS (*m/z* 419.0984 $[M-H]^-$) and ^{13}C NMR data. The ESI MS spectrum of **1** showed a quasimolecular ion peak at *m/z* 419 $[M-H]^-$ and one peak at *m/z* 287 $[(M-132)-H]^-$, due to the loss of one pentose moiety. Moreover, HR ESIMS/MS analysis revealed fragment ions at *m/z* 151.0058 $[C_7H_4O_4-H]^-$ and 135.0049 $[C_7H_4O_3-H]^-$, diagnostic for flavanoids carrying two hydroxyl groups on the A ring (de Rijke *et al*., 2006). The UV absorption bands at 281 and 327 nm were suggestive of a flavanone skeleton. The ^1H and ^{13}C NMR spectra (**Table 6.2**) displayed signals for an oxygenated methine doublet of doublets at δ 5.46 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), two methylene doublet of doublets at δ 3.14 (1H, dd, *J* = 17.0, 12.5 Hz, H-3_{ax}) and 2.82 (1H, dd, *J* = 17.0, 3.0 Hz, H-3_{eq}) and five aromatic protons of which two superimposable, that were assigned with the help of 1D TOCSY and DQF COSY, together with signals of a sugar residue. The chemical shifts of the two doublets at δ 7.40 (1H, d, *J* = 8.0 Hz, H-5) and 6.89 (1H, d, *J* = 8.0 Hz, H-6) suggested a 7,8-disubstituted A-ring, while the three signals at δ 7.01 (1H, d, *J* = 2.0 Hz, H-2'), 6.89 (1H, dd, *J* = 8.5, 2.0 Hz, H-6'), and 6.81 (1H, d, *J* = 8.5 Hz, H-5') were in accordance with a 1,2,4-trisubstituted B-ring. The twenty ^{13}C NMR resonances (**Table 6.2**) were easily assigned to a flavanone aglycone

moiety and to an α-arabinose unit (δ_C 102.7, 73.1, 71.4, 68.9, and 66.6). These 1D NMR data, in combination with the observed 2D NMR correlations, suggested that compound **1** was a flavanone having isookanin as aglycone (Agrawal, 1989). The position of the arabinose residue was deduced by the HMBC correlation between δ 4.97 (H-1_{ara}) and 151.8 ppm (C-7). Hydrolysis of **1** with 1N HCl, followed by GC analysis through a chiral column of the trimethylsilylated sugar, led to the assignment of arabinose configuration. The stereochemistry of C-2 was determined as *S* on the basis of a negative Cotton effect at 274 nm and a positive Cotton effect at 306 nm in the CD spectrum of **1**. Consequently, **1** was characterized as (2*S*)-isookanin 7-*O*-α-L-arabinopyranoside.

The molecular formula of compound **2** ($C_{22}H_{22}O_{11}$) was established by ^{13}C NMR and HR ESIMS spectra (*m/z* 461.1090 for $[M-H]^-$). In the HR ESIMS/MS spectrum fragments at *m/z* 401.1067 $[(M-60)-H]^-$, 287.0865 $[(M-174)-H]^-$, 151.0043 $[C_7H_4O_4-H]^-$ and 135.0041 $[C_7H_4O_3-H]^-$ were also observed. Its NMR spectral data (**Table 6.2**) suggested that the structure of **2** resembled that of **1**, but differed for the presence of an additional acetyl group. The ^{13}C NMR data (**Table 6.2**) were consistent with the presence of another isookanin glycoside, suggesting that the acetyl moiety should be linked to the arabinose residue. The acylation site was on C-2 of arabinose as evidenced by the strong deshielding of H-2_{ara} at δ 5.30 and was confirmed by HMBC experiment (Sun *et al.*, 2007). The stereochemistry at C-2 and the sugar unit configuration were determined as reported for **1**. Thus, the structure of **2** was deduced to be (2*S*)-isookanin 7-*O*-(2''-acetyl)-α-L-arabinopyranoside.

Compound **6** ($C_{27}H_{30}O_{16}$) showed a quasimolecular ion peak at *m/z* 609.1461 $[M-H]^-$ in the negative HR ESIMS. The prominent fragment ions observed in the ESI MS spectrum at *m/z* 447 $[(M-162)-H]^-$ and 285 $[(M-162-162)-H]^-$ together with the UV absorption maxima at 270 and 335 nm suggested the presence of a flavone glycoside. Analysis of the ^1H and ^{13}C NMR spectra (**Table 6.2**) of **6** clearly indicated that it was a luteolin derivative. The ^{13}C NMR spectrum allowed to assign 15 signals to the aglycone moiety and 12 to the sugar residue, consisting of one inner β-galactose and one terminal β-glucose. The linkage between sugar moieties was deduced by HMBC correlation between the signal at δ 4.39

(1H, d, J = 7.8 Hz, H-1_{glc}) and 69.3 (C-6_{gal}). Hydrolysis of **6** with 1N HCl, followed by GC analysis through a chiral column of the trimethylsilylated monosaccharides, permit the assignment of sugars configuration. Accordingly, compound **6** was assigned as luteolin 7-*O*-β-D-glucopyranosyl-(1→6)-β-D-galactopyranoside.

Compounds **3**-**4** and **7**-**11** were characterized as isookanin 7-*O*-β-D-glucopyranoside or flavanomarein (**3**) (Shimokoriyama, 1957), 8-methoxybutin (**4**) (Okada *et al*., 2014), butin 7-*O*-β-D-glucopyranoside or isocoreopsin (**5**) (Gupta *et al*., 1970), luteolin (**7**) (Agrawal, 1989), chrysoeriol 7-*O*-β-D-glucopyranoside or thermopsoside (**8**) (Romussi *et al*., 1996), eriodictyol 7-*O*-β-D-glucopyranoside (**9**) (Agrawal, 1989), 6',6''-sucrose ester of (1α,2α,3β,4β)-3,4-bis-(4-hydroxyphenyl)-1,2-cyclobutanedicarboxilic acid (**10**) (Wang *et al*., 2003), and chlorogenic acid (**11**) (Iwai *et al*., 2004) by spectrometric and spectroscopic data measurement and comparison with the literature data.

Finally, the biological activity of these new and known compounds using BCB, FRAP, and DPPH tests was measured (**Table 6.1**). Among known compounds luteolin (**7**) showed the highest values: 819.5 mgTE/g, 40.6% of antioxidant activity (AA), 1442.3 mgTE/g in DPPH, BCB and FRAP tests, respectively. These findings are congruent with previous evidences (Choi *et al*., 2014; Zhang *et al*., 2014). On the other hand, among new natural compounds, **1** showed to be the most active in DPPH and FRAP tests. On the basis of RACI (**Figure 6.3b**), that reflects the results obtained from all antioxidant tests, it is possible to assess that **7** and **1** are the most active compounds followed by compound **11** and a group formed by compounds **9**, **4**, **3, 6,** and **2.** This group showed close but slightly descending RACI values. The lowest RACIs were observed for compounds **5**, **8** and **10**. All isolated compounds are flavonoid derivatives with the exclusion of **10** and **11**, and on the basis of their structures it is possible to confirm the importance of the adjacency of the two hydroxyl groups in the *ortho*-diphenolic arrangement (compounds **7**, **1**, **11**, **9**, **4**, **3**, **6**, **2**). The loss of activity of compound **8** could be ascribed to the presence of a methoxy group on the B-ring. This result is congruent with previous findings where it was demonstrated that the presence of a methoxy group decreases sensibly the antioxidant capacity of flavonoids (Heim *et al*., 2002; Rice-Evans *et al*., 1996). Some of the pure compounds tested

exerted interesting antioxidant activity, and their action could partially explain the activity shown by the fraction they come from. Thus, in order to deepen the polar fraction metabolome of *B. humilis*, the *n*-BuOH extract was subjected to LC-HRESIMS and LC-HRESIMS/MS analyses both in positive and in negative ion mode (**Figure 6.4**). Twenty-four major compounds were detected including the eleven isolated compounds; on the basis of accurate molecular weight determination, and taking into account the main fragments observed, the structure of further thirteen derivatives was proposed (**Table 6.3**). The presence of these additional phenolic compounds could explain the antioxidant activity of *B. humilis n*-BuOH extract.

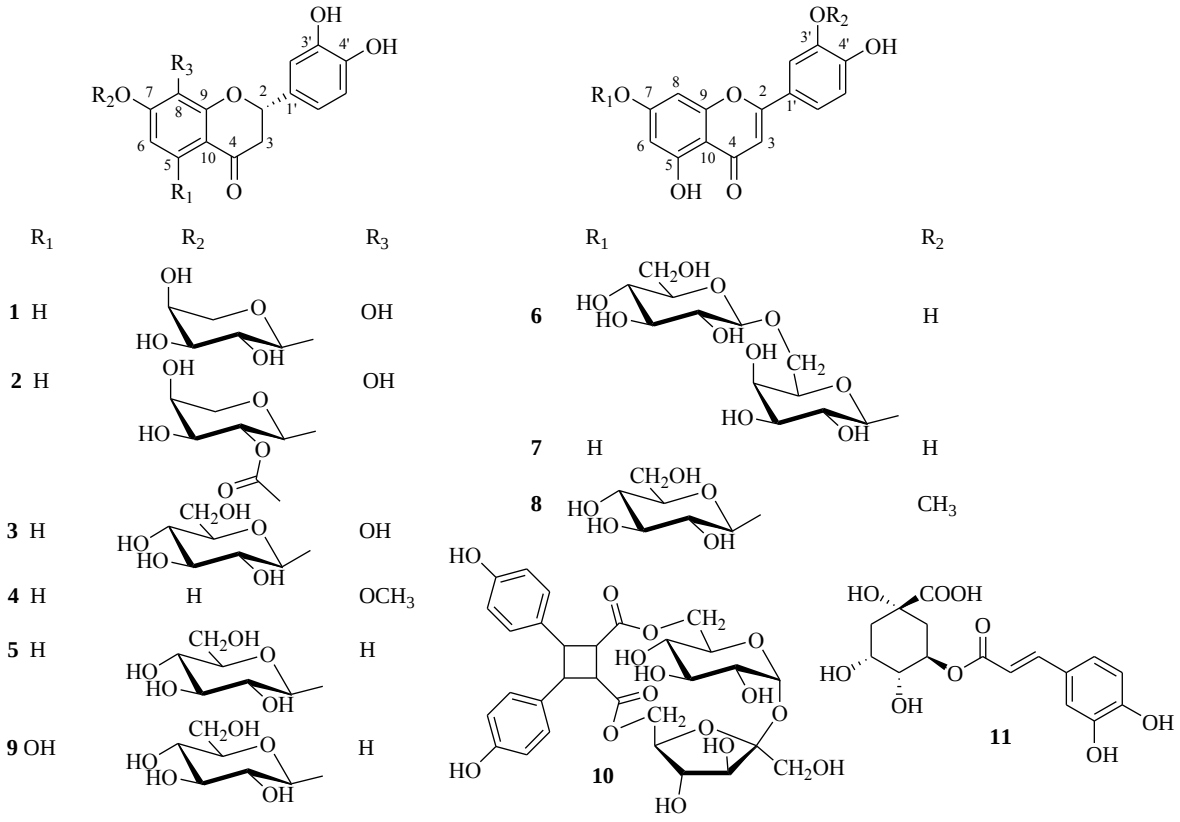
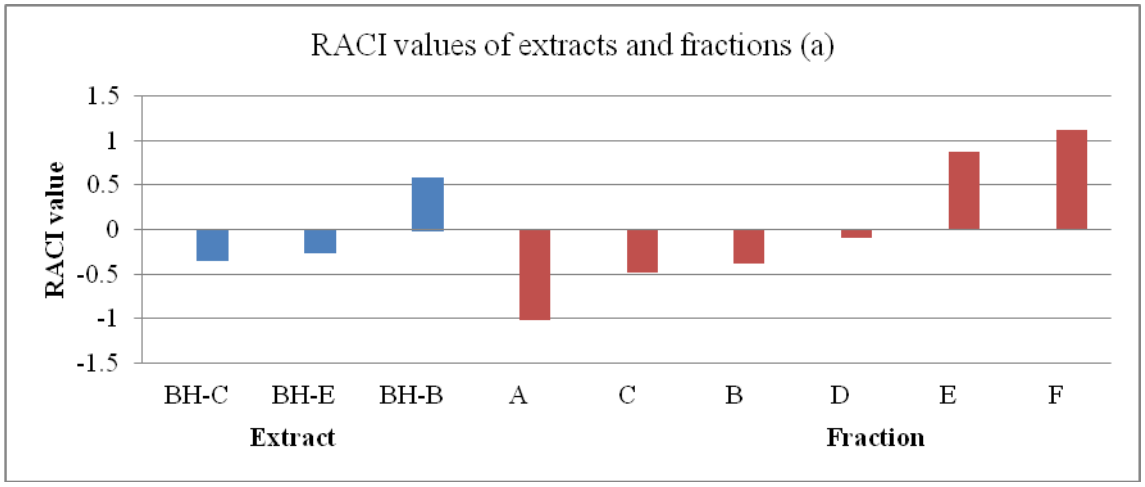


Figure 6.2: Chemical structures of compounds **1-11**



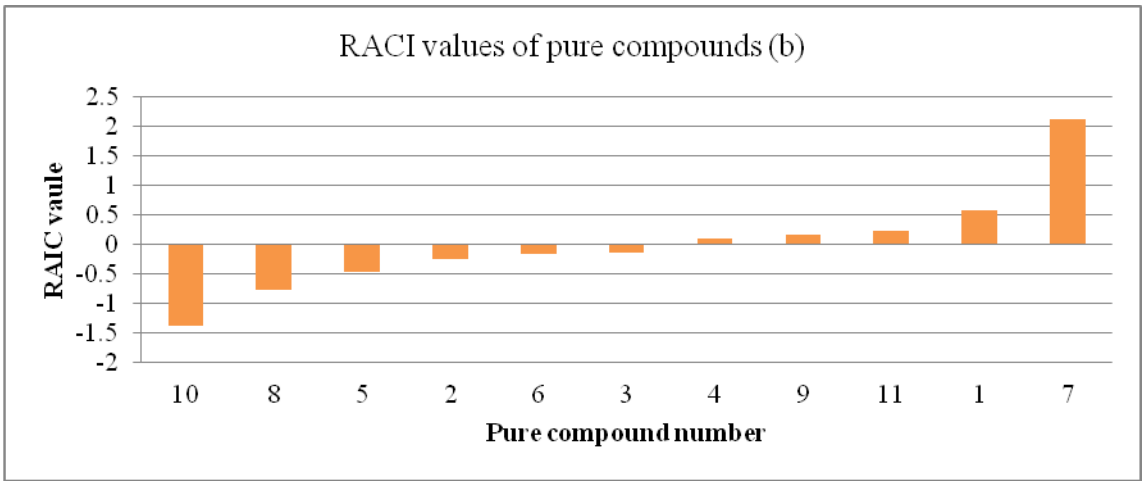


Figure 6.3: Relative Antioxidant Capacity Index (RACI) values of extracts (BH-C: chloroform; BH-E: *n*-hexane; BH-B: *n*-BuOH), Sephadex-LH20 fractions (indicated with letters) (a), and pure compounds (indicated with numbers) (b) obtained from *Bidens humilis* aerial parts.

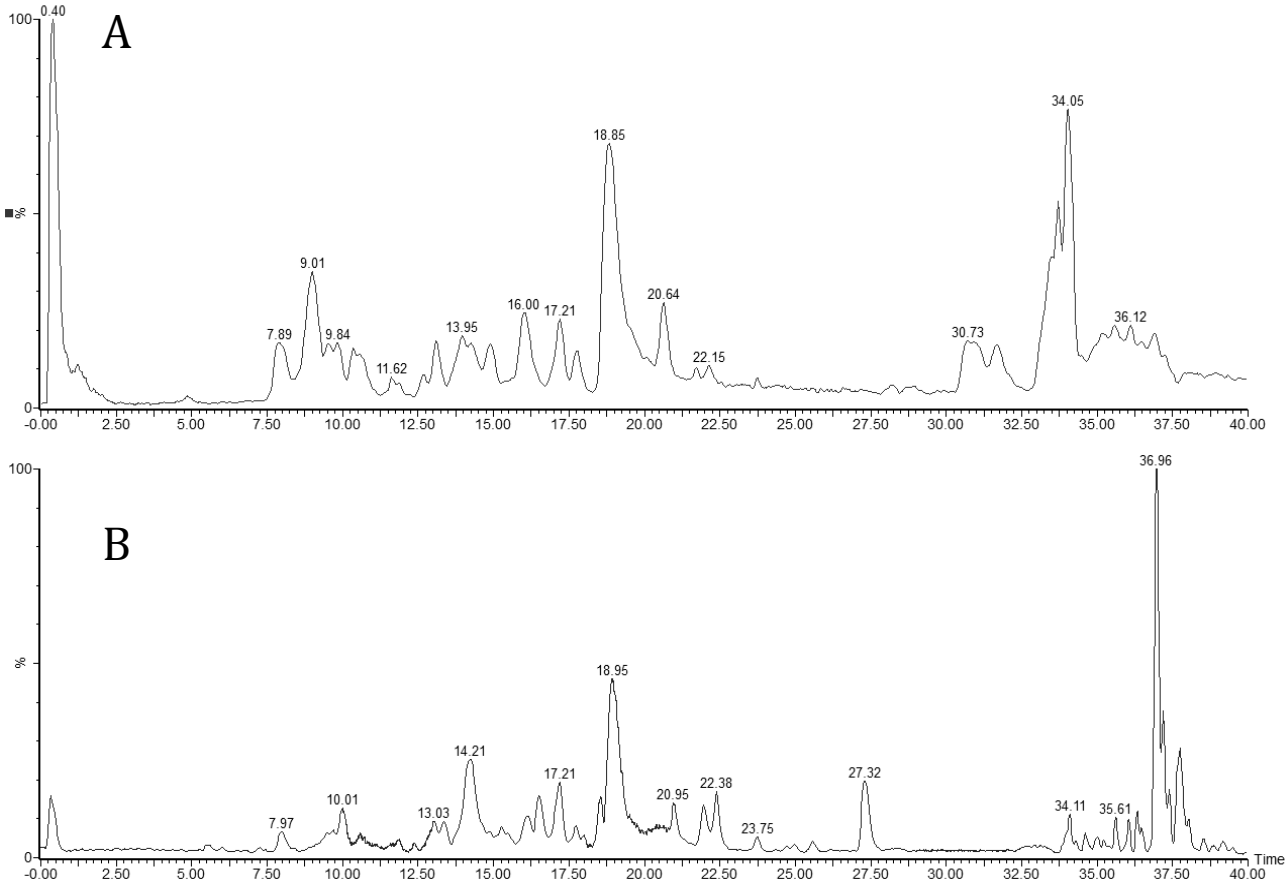


Figure 6.4: Total ion current LC-HRESIMS chromatograms obtained for the *n*-BuOH fraction of the aerial parts of *B. humilis* in negative (A) and in positive (B) ion mode.

Table 6.1. Antioxidant activity and total phenolic content of *B. humilis* extracts and fractions using DPPH, BCB, FRAP, and Folin assays.

Sample	**TEST**			
Extract	*DPPH mgTE/g**	*BCB %AA***	*FRAP mgTE/g**	*Folin mgGAE/g****
***n*-hexane**	34.9±1.7	43.6±2.9	6.5±0.5	34.0±2.2
$CHCl_3$	37.6±2.4	37.9±1.9	31.3±2.1	13.3±0.9
***n*-BuOH**	173.8±6.2	8.0±0.7	790.1±12.5	261.7±11.0
Fraction				
A	27.4±1.0	13.8±0.4	115.0±9.2	90.1±5.2
C	163.8±8.5	9.4±0.2	620.7±19.5	361.8±22.2
B	147.1±11.2	3.2±0.4	675.1±23.1	451.0±21.3
D	296.3±14.5	4.5±0.5	954.3±12.7	495.5±24.2
E	857.3±18.8	11.4±0.7	2208.8±45.2	511.1±19.7
F	819.5±21.2	40.6±1.1	1442.3±32.1	518.0±16.5
Pure compound				
8	151.5±3.2	9.5±0.7	170.5±4.5	
10	317.5±12.2	14.4±0.9	297.5±7.4	
5	238.0±7.7	23.2±1.1	421.2±9.2	
2	484.7±21.3	10.3±0.8	777.6±12.4	
6	357.8±17.5	17.1±1.0	823.8±11.4	
3	356.5±16.4	20.5±1.5	707.4±12.6	
4	255.3±12.9	22.3±1.1	1129.7±24.0	
9	477.1±14.5	21.0±0.9	770.8±7.0	
11	470.7±21.6	19.3±1.7	953.1±12.9	
1	513.7±28.7	16.8±1.0	1404.2±15.6	
7	819.5±22.5	40.6±2.1	1442.3±17.8	

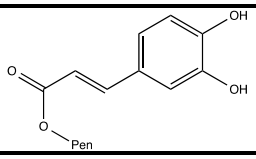
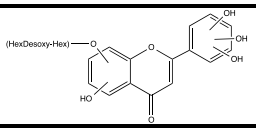
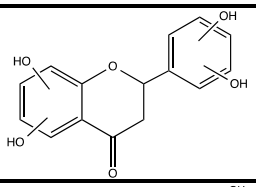
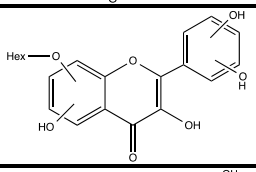
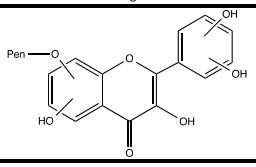
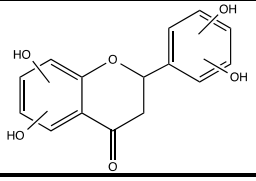
Values are the mean of three determinations ($P < 0.05$). *Milligrams of Trolox Equivalents per g of Extract/Fraction/Pure compound; **Antioxidant activity at [0.05 mg/mL]; *** Milligrams of Gallic Acid Equivalents per g of Extract/Fraction/Pure compound.

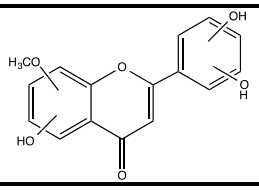
Table 6.2. ^{1}H and ^{13}C-NMR data of compounds **1**, **2**, and **6** (CD_3OD, 600 MHz, *J* in Hz)

position	**1**		**2**		**6**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	5.46 dd (12.5, 3.0)	81.0	5.43 dd (12.8, 3.0)	81.4		166.0
3ax	3.14 dd (17.0, 12.5)	45.0	3.14 dd (17.2, 12.8)	44.6	6.62 s	103.1
3eq	2.82 dd (17.0, 3.0)		2.77 dd (17.2, 3.0)			
4		193.0		193.3		182.9
5	7.40 d (8.0)	118.0	7.38 d (8.2)	118.0		161.4
6	6.89 d (8.0)	110.0	6.88 d (8.2)	111.3	6.62 d (2.0)	101.7
7		151.8		150.6		163.0
8		135.6		136.3	6.83 d (2.0)	95.2
9		155.0		155.4		157.8
10		113.9		113.0		105.9
1'		130.6		130.5		122.3
2'	7.01 d (2.0)	114.5	7.02 d (2.0)	114.9	7.45 d (2.0)	113.2
3'		145.0		145.0		145.9
4'		145.9		145.8		150.0
5'	6.81 d (8.5)	115.7	6.81 d (8.5)	115.9	6.94 d (8.0)	115.6
6'	6.89 dd (8.5, 2.0)	118.9	6.88 dd (8.5, 2.0)	119.2	7.45 dd (8.0, 2.0)	119.5
Ara 1	4.97 d (7.0)	102.7	5.17 d (6.8)	100.6		
2	3.92 dd (9.0, 7.0)	71.4	5.30 dd (9.0, 6.8)	73.0		
3	3.70 dd (9.0, 2.5)	73.1	3.88 dd (9.0, 2.5)	71.6		
4	3.94 m	68.9	3.94 m	68.6		
5a	3.95 dd (12.0, 2.0)	66.6	3.98 dd (12.0, 2.0)	66.7		
5b	3.74 dd (12.0, 3.5)		3.72 dd (12.0, 3.5)			
$\underline{CO}CH_3$				171.3		
$CO\underline{CH_3}$			2.12 s	21.0		
Gal 1					5.09 d (7.5)	100.5
2	-				3.52 dd (9.0, 7.5)	73.9
3					3.53 dd (9.0, 4.0)	76.3
4					3.47 dd (4.0, 2.5)	70.3
5					3.83 m	76.6
6a					4.23 dd (12.0, 2.5)	69.3
6b					3.87 dd (12.0, 4.5)	
Glc1					4.39 d (7.8)	103.9
2					3.30 dd (9.0, 7.8)	74.0
3					3.29 t (9.0)	77.4
4					3.45 t (9.0)	70.6
5					3.37 m	77.0
6a					3.91 dd (12.0, 3.0)	62.0
6b					3.69 dd (12.0, 5.0)	

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments

Table 6.3. Identification of the major compounds observed in the HR-LCMS analysis of *B. humilis* extract both in negative or positive ion mode (**Figure 6.3**).

R.t. (min)	Experimental MW	Positive ion mode (*m/z*)	Negative ion mode (*m/z*)	Main fragments (*m/z*)	Attempted id./compound	Theoretical MW
0.4	312.1053		311.0874	179.07, 139.06, 135.06, 131.05, 89.01		312.0845
0.5	354.1002		353.0923	191.04, 179.04, 135.03	**11**	354.0951
7.8	516.1309		515.123	353.09, 191.07, 179.06, 135.03	Dicaffeoylquinic acid	516.1268
8	610.1576		609.1497	447.11, 301.09, 151.01		610.1534
9.8	450.1186		449.1107	287.09, 151.01, 135.01	**3**[1]	450.1162
	288.0719	289.0696	287.0743	179.01, 151.01, 135.01		288.0634
10.7	464.1200	465.1005	463.1395	301.07, 179.01, 151.01, 135.01		464.0955
13	434.0940		433.0862	303.05, 151.01, 135.01		434.0849
13.1	610.1595	611.1603	609.1587	447.11, 285.10, 151.01, 135.00	**6**	610.1534
13.9	450.1168		449.1089	287.08, 151.01, 135.01	**9**[1]	450.1162
14.9	420.1087		419.1008	287.09, 151.01, 135.01	**1**	420,1056
16	288.0718	289.0696	287.0741	179.01, 151.01, 135.01		288.0624
17.2	462.1183	463.1129	461.1237	446.13, 299.04, 285.04, 151.05	**8**	462.1162

	300.0695	301.0743	299.0648	284.06, 165.01, 151.09, 135.01		300.0634
17.8	434.1245		433.1166	271.05, 125.01	**5**	434.1213
18.8	462.1170	463.1167	461.1174	401.10, 287.09, 151.01, 135.01	**2**	462.1162
	634.1905		633.1826	487.18, 353.17, 293.09, 163.01, 145.01	**10**	634.1898
	286.0569	287.0403	285.0736	151.01, 135.01	**7**	286.0477
20.6	302.0852	303.0833	301.0871	286.08, 165.01	**4**	302.0790
	530.1568		529.1489	367.13, 301.10, 287.09, 179.07, 161.05, 131.01, 135.01		
22.1	676.2078		675.1999	463.16, 403.12, 211.11, 87.01		
27.4	824.3918	413.2037 $[M+2H]^{2+}$		678.36, 621.28, 474.25, 276.18, 148.00	Peptide (FGAVVFGE)	824.4069
30.7	448.0892	449.0842	447.0943	285.09, 151.01, 135.01	Glycosil-luteolin	448.1006
31.7	554.0938		553.0859	419.04, 413.03	Dimeric-luteolin	554.0849
	326.2112		325.2033	255.10, 183.06, 97.00		
34.0	340.2192		339.2113	269.10, 183.07, 97.00		
35.6	286.0577		285.0498	151.01, 135.01	Luteolin	286.0477
	254.0631		253.0552	151.01, 135.01	Chrysin	254.0579
36.9	370.0548	371.0627		259.06, 127.01		
37.8	426.1431	427.151		259.07, 127.03		

[1] This identification was confirmed by pure compound injection
pen = pentose
hex = hexose

CHAPTER 7

Phytochemical study of *Andromachia igniaria* Humb. & Bonpl

Chapter 7

7.1 Introduction: *Andromachia* genus

In 1809 Bonpland described, in his publication "Plantes Equinoxiales", twelve new species from Ecuador. Within this group maybe the most interesting species correspond to *Andromachia igniaria* Humb. & Bonpl.(**Figure 7.1**). *Andromachia* is a genus of flowering plants in the Asteraceae family, which was recognized in 1819 as a synonym of *Liabum* and placed in the Vernonieae (Brettell *et al*., 1973). This species was found for the first time near Quito - Ecuador, in a zone called Valle de los Chillos. The name "*igniaria*", that means "of fire", comes from the intense yellow color of the inflorescences. The species is widely distributed in the Andes mainly from Colombia and Ecuador, between 2000 and 3500 m a.s.l., and has also the synonym *Liabum igniarum* Less. It is a shrub, 1-3 m high, with bracts separated by petioles, opposite petiolated leaves, with abundant white pubescent in the underside, top inflorescence, ligulate yellow flower and the fruit is an achene. The plant is very used by peasants that take advantage of the bark to make fire to cook or to warm up homes. Leaves are also used to feed guinea pigs, as well as to prepare an special sweet bread called "pan de leche". In popular medicine people use the leaves to wash the body for purification, to treat headache or to heal wounds.

Few informations are reported about previous investigations carried out on some species of *Andromachia* genus, from Central and South America, that result basically on the isolation of some simple sesquiterpene lactones, triterpenes, and flavonoids (Bohlmann *et al*., 1984; Jakupovic *et al*., 1988; Juarez *et al*., 1995).

Figure 7.1: *Andromachia igniaria*

7.2 Plant material

Aerial parts of *A. igniaria* were collected in Tumbaco, Ecuador in September 2011. The plant was identified at the Herbarium of Jardin Botanico de Quito, Quito, Ecuador. A voucher specimen (N. 9371 Andromachia igniaria /1) was deposited at Herbarium Horti Botanici Pisani, Pisa, Italy.

7.3 Extraction and isolation

The dried and powdered aerial parts (580 g) of *A. igniaria* were in sequence extracted for 48 h with *n*-hexane, $CHCl_3$, $CHCl_3$-MeOH (9:1) and MeOH, by exhaustive maceration (3 x 2 L), to give 8.2, 14.9, 6.3 and 23.1 g of the respective residues.

The MeOH extract was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH residue. The *n*-BuOH fraction (4 g) was submitted to Sephadex LH-20 column (3 x 100 cm, flow rate 1.0 mL/min) using MeOH as eluent to obtain sixteen major fractions (A-P) grouped by TLC. Fraction G (109.2 mg) was subjected to RP-HPLC with MeOH-H_2O (45:55) as eluent yielding a new flavonoid 3',4',7,8-tetrahydroxyflavanone 7-*O*-(6''-O-acetyl)-β-D-

glucopyranoside (compound **1**, 4.3 mg, t_R = 10 min), and caffeic acid metil ester (compound **2**, 1.9 mg, t_R = 14 min), rutin (compound **3**, 2.7 mg, t_R = 15 min) and kaempferol 3-*O* -β-D-glucopyranoside (compound **4**, 0.9 mg, t_R = 21 min) . Fractions A (215.2 mg), B (136.8 mg), N (20.3 mg) and P (25 mg) were separately purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to give 3-β-D-glucopyranosyloxy-1-hydroxy-6(E)-tetradecene 8,10,12-triyne (compound **5**, 2.2 mg, t_R = 35 min), eugenyl *O*-β-D-glucopyranoside (compound **6**, 1.5 mg, t_R = 10 min) from fraction A, 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (compound **7**, 0.8 mg, t_R = 38 min) from B, eriodictyol (compound **8**, 1.9 mg, t_R = 12 min) from N, finally okanin (compound **9**, 1.4 mg, t_R = 13 min), butein (compound **10**, 17.3 mg, t_R = 22 min) and luteolin (compound **11**, 0.3 mg, t_R = 25 min) from fraction P. Fractions E (70.9 mg), I (143.3 mg), J (424.9 mg) and L (67.9 mg) were also subjected to to RP-HPLC with MeOH-H_2O (45:55) as eluent to obtain bidenoside F (compound **12**, 1.3 mg, t_R = 13 min) from E, compound **4** (1.8 mg, t_R = 20 min), quercetin 3-*O*-β-D-glucopyranoside (compound **13**, 5.3 mg, t_R = 15 min) from J, okanin 4'-*O*-β-D-glucopyranoside (compound **14**, 14.2 mg, t_R = 12 min) and luteolin 4'-*O*-β-D-glucopyranoside (compound **15**, 1.1 mg, t_R = 23 min) from fraction L.

The $CHCl_3$-MeOH extract (6 g) was subjected to Sephadex LH-20 column (3 x 100 cm, flow rate 1 mL/min) eluting with MeOH to give eleven major fractions (A-K) grouped by TLC, together with pure luteolin (compound **11**, 11.6 mg). Fraction E (232 mg) was purified by RP-HPLC with MeOH-H_2O (55:45) as eluent to afford compound **5** (1 mg, t_R = 19 min). Fraction G (130.7 mg) was purified by RP-HPLC with MeOH-H_2O (4:6) as eluent yielding compound **1** (1 mg, t_R = 16 min). Fraction H (108 mg) was subjected to RP-HPLC with MeOH-H_2O (45:55) as eluent to give compound **1** (1.1 mg, t_R = 10 min) and caffeic acid (compound **16**, 5.3 mg, t_R = 6 min). Finally fraction I (34.1 mg) was purified by RP-HPLC with MeOH-H_2O (1:1) as eluent to afford a new flavonoid 3',4',7,8-tetrahydroxyflavone 7-*O*-(6"-*O*-acetyl)-β-D-glucopyranoside (compound **17**, 2 mg, t_R = 18 min).

The structures of new compounds are shown in **Figure 7.2**. The structures of the other knowm compounds are reported in **Figures 7.3-7.5**.

7.4 New isolated compounds

3',4',7,8-tetrahydroxyflavanone 7-*O*-(6"-*O*-acetyl)-β-D-glucopyranoside (**1**)

Yellow amorphous powder; $[\alpha]_D^{25}$ -40 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε): 328 (4.19), 284 (3.90); CD $[\theta]_{25}$ (*c* 0.05, MeOH, nm) – 5000 (276 nm), + 4520 (304 nm); ^{1}H and ^{13}C NMR data, see **Table 7.1**; ESI MS *m/z* 491 $[M-H]^-$, 473 $[M-H-18]^-$, 287 $[M-H-204]^-$, HR ESIMS $[M-H]^-$ 491.1182 (calcd for $C_{23}H_{23}O_{12}$ 491.1190), 355.1042 $[M-H-134]^-$, 287.0993 $[M-H-204]^-$.

3',4',7,8-tetrahydroxyflavone 7-*O*-(6"-*O*-acetyl)-β-D-glucopyranoside (**17**)

Yellow amorphous powder; $[\alpha]_D^{25}$ -35 (*c* 0.078, MeOH); UV (MeOH) λ_{max} (log ε): 258 (4.07), 321 (3.85); ^{1}H and ^{13}C NMR data, see **Table 7.1**; ESI MS *m/z* 489 $[M-H]^-$; HR ESIMS *m/z* 489.1041 $[M-H]^-$ (calcd for $C_{23}H_{21}O_{12}$ 489.1033), 355.0972 $[M-H-134]^-$, 285.0944 $[M-H-204]^-$.

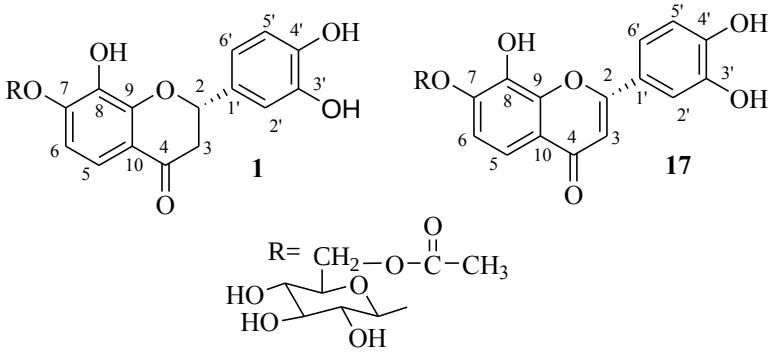


Figure 7.2: Structures of compounds **1** and **17**

7.5 Structural elucidation

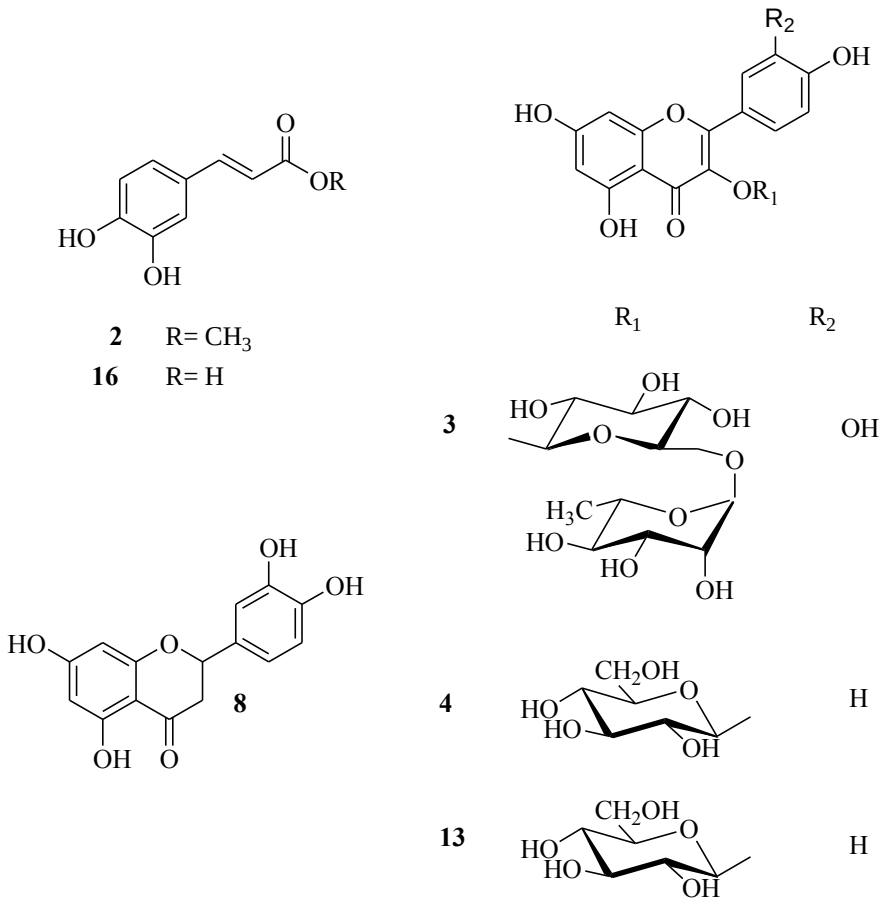
Compound **1** was obtained as a yellow powder, analyzed for the molecular formula $C_{23}H_{24}O_{12}$ by HRESI-MS at *m/z* 491.482 $[M-H]^-$ (calcd for $C_{23}H_{23}O_{12}$, 491.1190), which was further confirmed by ^{13}C NMR and DEPT spectra. The ESI MS spectrum of compound

1 showed a quasimolecular ion peak at *m/z* 491 $[M-H]^-$ and one peak at *m/z* 287 $[M-H-204]^-$, due to the loss of one acetylated hexose moiety. The UV absorption bands at 328 and 284 nm were suggestive of a flavanone skeleton. In the ^1H NMR spectrum, three aromatic protons signals (δ 7.04, d, *J* = 2.0 Hz; δ 6.92, dd, *J* = 8.5, 2.0 Hz; δ 6.82, d, *J* = 8.5 Hz) indicated an ABX system on ring B, and these proton signals were assigned to H-2', H-6' and H-5' by HSQC analysis. Likewise, another two aromatic protons signals (δ 7.40, d, *J* = 8.0 Hz; δ 6.90, d, *J* = 8.0 Hz) were readily assigned to H-5 and H-6 of ring A. The ^1H and ^{13}C NMR spectra (**Table 7.1**) displayed also signals for an oxygenated methine doublet of doublets at δ 5.45 (1H, dd, *J* = 12.0, 3.0 Hz, H-2), two methylene doublet of doublets at δ 3.16 (1H, dd, *J* = 17.0, 12.0 Hz, H-3_{ax}) and 2.81 (1H, dd, *J* = 17.0, 3.0 Hz, H-3_{eq}), that were assigned with the help of 1D TOCSY and DQF COSY, together with signals of a sugar residue and an acetyl group. These 1D NMR data, in combination with the observed 2D NMR correlations, suggested that compound **1** was a flavanone having isookanin as aglycone (Agrawal, 1989). The structure of the monosaccharide moiety of compound **1** was deduced using hydrolysis followed by trimethyl silylation and GC-analysis, 1D-TOCSY and DQF-COSY experiments. Thus, the chemical shifts of the sugar resonances were attributable to one β-glucopyranosyl unit esterified at C-6 position. The substitution sites of the glucose and acetyl residues were also confirmed by the HMBC correlations between δ 4.98 (H-1_{glc}) and 152.2 ppm (C-7) and δ 4.45, 4.28 (H_2-6_{glc}) and 172.0 ppm ($COCH_3$). The stereochemistry of C-2 was determined as *S* on the basis of a negative Cotton effect at 276 nm and a positive Cotton effect at 304 nm in the CD spectrum. Thus, the structure of compound **1** was unambiguously elucidated as 3',4',7,8-tetrahydroxyflavanone 7-*O*-(6"-O-acetyl)-β-D-glucopyranoside or 6"-O-acetyl-flavanomarein.

The molecular formula of compound **17** ($C_{23}H_{22}O_{12}$) was established by ^{13}C NMR and HR ESIMS spectra (*m/z* 489.104 for $[M-H]^-$). Its NMR spectral data suggested that the acetylated sugar moiety of compound **17** was superimposable to that of compound **1**, while the aglycone was the point of difference. In fact, ^1H and ^{13}C NMR spectra (**Table 7.1**) revealed the presence of an additional aromatic signal (δ_H 6.78 s, δ_C 115.5), which could be easily assigned to ring C, and was consistent with the presence of 3',4',7,8-

tetrahydroxyflavone as aglycone (Yang *et al*., 2012). The configuration of the sugar moiety was determined as reported for compound **1**. Thus, the structure of **17** was elucidated as 3',4',7,8-tetrahydroxyflavone 7-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside.

The following known compounds were identified by spectral analysis and comparison with published spectroscopic data: caffeic acid methyl ester (**2**), caffeic acid (**16**) (Saleem *et al.*, 2004), rutin (**3**) (Agrawal, 1989), kaempferol 3-*O*-β-D-glucopyranoside (**4**), 3-β-D-glucopyranosyloxy-1-hydroxy-6(E)-tetradecene 8,10,12-triyne (**5**) (Rücker *et al*., 1992), eugenyl *O*-β-D-glucopyranoside (**6**) (Fujita *et al*., 1994), 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (**7**) (Chiang *et al*., 2007), eriodictyol (**8**) (Agrawal, 1989), okanin (**9**), butein (**10**) (Chokchaisiri *et al*., 2009), luteolin (**11**), bidenoside F(**12**) (Li *et al*., 2005), quercetin 3-*O*-β-D-glucopyranoside (**13**) (Agrawal, 1989), okanin 4'-*O*-β-D-glucopyranoside (14) (Hoffmann and Bhölzl, 1988) and luteolin 4'-*O*-β-D-glucopyranoside (**15**) (Nawwar *et al.*,1994).



2 R= CH_3

16 R= H

Figure 7.3: Structures of known compounds from *A. igniaria*

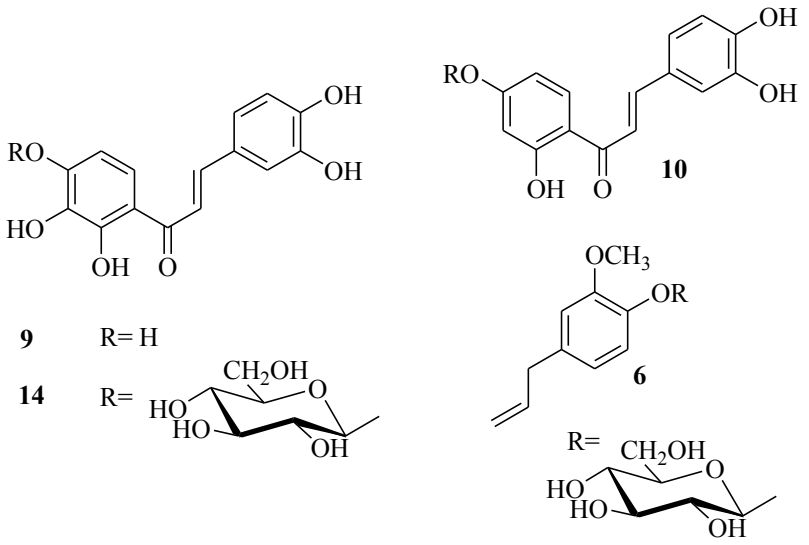


Figure 7.4: Known compounds isolated from *A. igniaria*

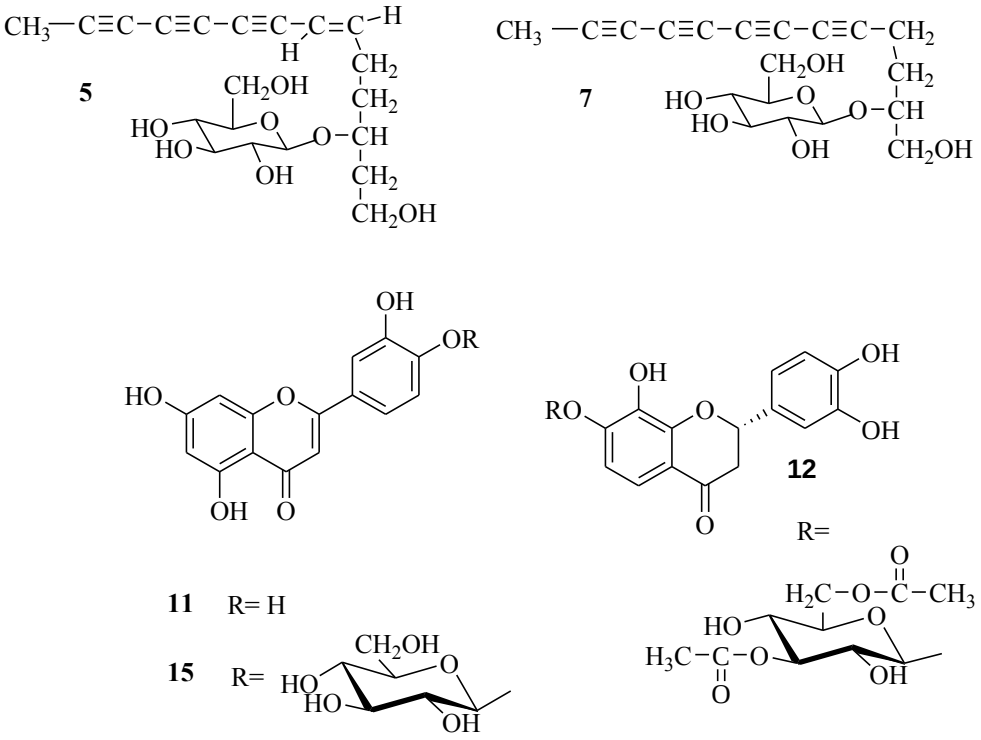


Figure 7.5: Known compounds isolated from *A. igniaria*

Table 7.1. ^{1}H and ^{13}C-NMR data of compounds **1** and **17** (CD_3OD, 600 MHz, *J* in Hz)

position	**1**		**17**	
	δ_H	δ_C	δ_H	δ_C
2	5.45 dd (12.0, 3.0)	81.5		154.0
3ax	3.16 dd (17.0, 12.0)	44.8	6.78 S	115.5
3eq	2.81 dd (17.0, 3.0)			
4		194.0		193.3
5	7.40 d (8.0)	118.3	7.22 d (8.0)	114.8
6	6.90 d (8.0)	111.0	7.09 d (8.0)	113.0
7		152.2		153.6
8		136.5		135.6
9		152.0		148.0
10		113.8		119.4
1'		131.5		125.6
2'	7.04 d (2.0)	115.3	7.60 d (1.8)	119.2
3'		145.0		147.7
4'		146.5		149.0
5'	6.82 d (8.5)	116.5	6.86 d (8.0)	116.7
6'	6.92 dd (8.5, 2.0)	118.6	7.39 dd (8.0, 1.8)	126.7
Glc 1	4.98 d (7.8)	102.6	5.00 d (7.8)	102.9
2	3.54 dd (9.0, 7.8)	74.7	3.60 dd (9.5, 7.8)	74.5
3	3.51 t (9.0)	77.6	3.50 t (9.5)	77.7
4	3.42 t (9.0)	71.6	3.43 t (9.5)	71.8
5	3.72 m	75.6	3.73 m	76.2
6a	4.45 dd (12.0, 3.0)	65.0	4.49 dd (12.0, 3.0)	65.0
6b	4.28 dd (12.0, 5.0)		4.30 dd (12.0, 4.5)	
$\underline{CO}CH_3$		172.0		172.7
$CO\underline{CH}_3$	2.14 s	20.8	2.10 s	21.1

Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments

CHAPTER 8

Conclusions

CONCLUSIONS

This thesis work reported the results of the phytochemical study of *Siparuna thecaphora* (Poepp. Et Endl) A.DC, *Bidens humilis* (Kunth), *Andromachia igniaria* (Humb.& Bonpl.), *Euphorbia laurifolia* (Juss. Ex Lam.), and *Clinopodium tomentosum* (Kunth) Harley. These plants belong to Ecuadorian flora and have not been studied before for their chemical content, nevertheless they are used in traditional medicine in Ecuador, and also belong to genus that demonstrated to be interesting according to information reported in the literature. Chromatographic separation led to the isolation of 60 compounds, elucidated by extensive spectroscopic and spectrometric methods, of which 13 are new (**Figures 8.1, 8.2, 8.3 and 8.4**) and 47 known and belong to different classes of secondary metabolites such as monoterpene, phenolics, diterpenes and flavonoids.

The detailed results of the research for each plant are reported in **Chapters 3-7**.

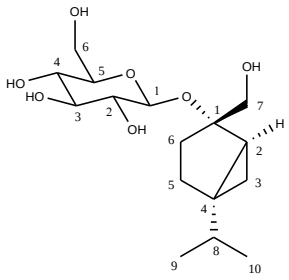


Figure 8.1: New isolated compound from *S. thecaphora*, see **Chapter 3**

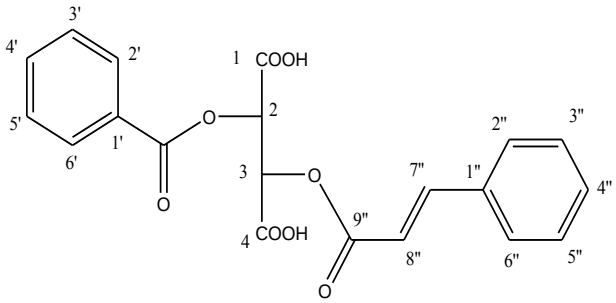


Figure 8.2: Structure of new compound isolated from *C. tomentosum*, see **Chapter 4**

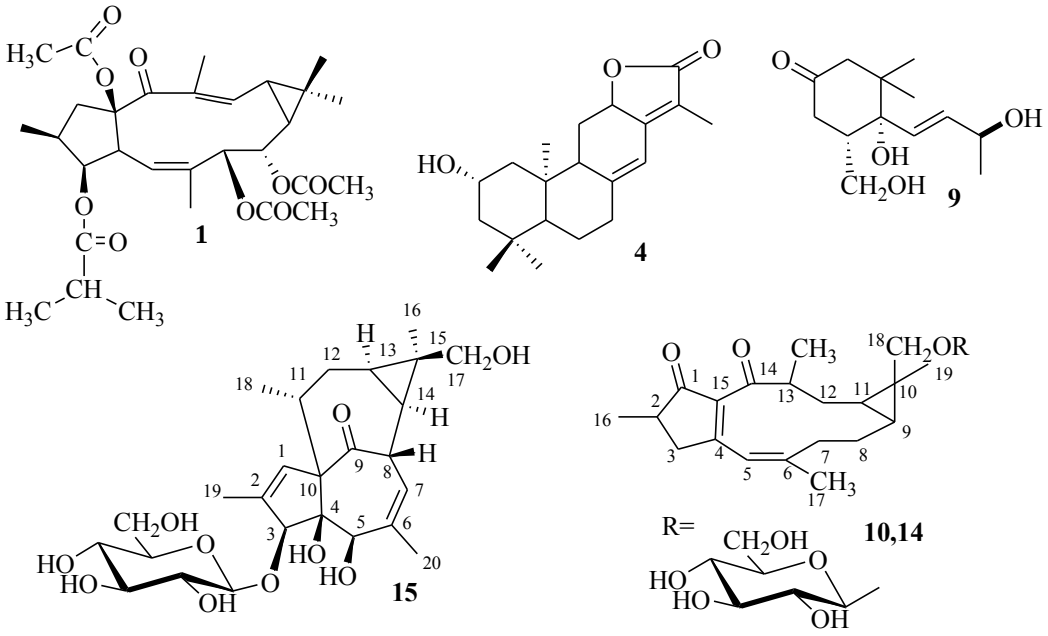


Figure 8.3: New isolated compounds from *E. laurifolia*, see **Chapter 5**

Since diterpenes from *Euphorbia* genus are reported to show important antitumor activity, the antiproliferative activity of compound **10** was preliminary assayed against human prostate cancer (PC-3) and human breast adenocarcinoma (MCF7) cell lines, showing an IC_{50} value of 25 µM at 24 and 48 h. In order to identify compound **10** molecular target a Drug Affinity Responsive Target Stability (DARTS) method was performed, and Clathrin heavy chain 1was found to be a possible target. These are preliminary results that will be confirmed and validated in a future work.

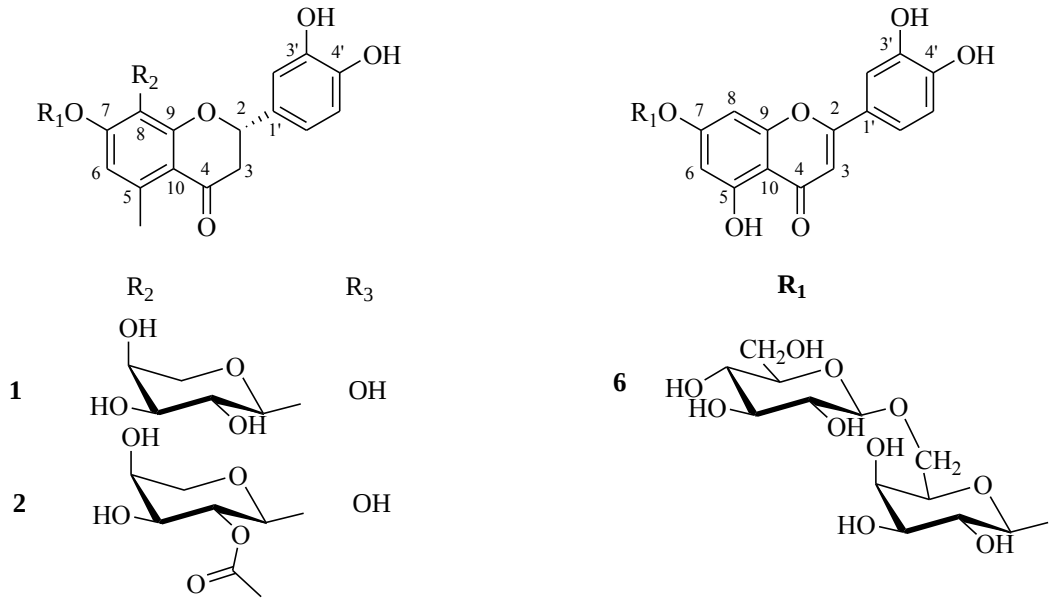


Figure 8.4: Structures of new compounds isolated from *B. humilis*, see **Chapter 6**

The 2,2-Diphenyl-2-picrylhydrazyl (DPPH), β-carotene bleaching (BCB) and ferric reducing antioxidant power (FRAP) assays were applied to all the extracts. Total Phenolic Content (TPC) was also measured in each case. The *n*-BuOH fraction demonstrated to be the one containing the highest concentration of total phenolics thus was subsequently submitted to chromatographic separation to obtain less complex fraction and finally isolate compounds that were also test by the same assays to screen their antioxidant activity. Most of the isolated compounds are flavonoids; considering their structure the leading role of two hydroxyl groups in the *ortho*-diphenolic could be responsible of the antioxidant activity. The presence of additional phenolic compounds (**Chapter 6**) detected thanks to LC-HRESIMS and LC-HRESIMS/MS analyses carried out with the *n*-BuOH extract could explain the antioxidant activity of *B. humilis n*-BuOH extract.

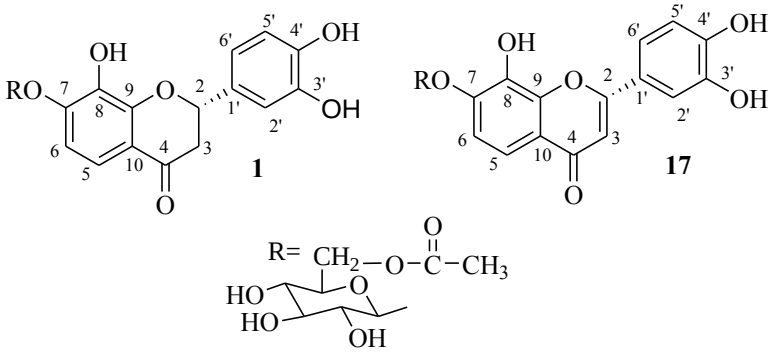


Figure 8.5: New compounds from *A. igniaria*, see **Chapter 7**

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LIST OF ABBREVIATIONS

AA: antioxidant activity

AcOEt: ethyl acetate

AcOH: acetic acid

acylCoA: coenzyme A

ATP: adenosine triphosphate

BCB: β-Carotene bleaching

BHT: butylhydroxytoluen

1D-2D: mono e bidimensional

CD: circular dichroism

CD_3OD: deuterate methanol

CH_3CN: acetonitrile

$CHCl_3$: Chloroform

^{13}C-NMR: carbon nuclear magnetic resonance

COSY: correlation spectroscopy

$CeSO_4$: ceric sulfate

C-terminal: carboxy-terminal

DAD: diode array detector

DARTS: Drug Affinity Responsive Target Stability

DEPT: Distortionless Enhancement by Polarization Transfer

DMSO: dimethyl sulfoxide

DPPH: sodium acetate trihydrate

DQF: double-quantum filtered

DTT: dithiothreitol

ESI-MS: electrospray mass spectrometry

FRAP: Ferric reducing antioxidant power

g: gram

GLC: glucose

HCl: chloridic acid

HMBC: heteronuclear multiple bond correlation

^{1}H-NMR: proton nuclear magnetic resonance spectroscopy

HPCPC: high performance centrifugal partition chromatography

HPLC: high pressure liquid chromatography

HR-MS: high-resolution mass spectrometry

HRESIMS: high resolution electrosray mass spectrometry

H_2SO_4: sulfuric acid

HSQC: heteronuclear single quantum correlation

Hz: hertz

IAA: iodoacetamide

IC_{50}: inhibitory concentration half maxima

J: NMR coupling costant

kDa: kilodaltons

KOH: potassium hydroxide

LC-MS: liquid chromatography coupled with mass spectrometry

MCF7: human breast adenocarcinoma cell line

m/z: mass/ charge

MeOH: methanol

mg: milligram

MHz: mega Hertz

MS: mass spectrometry

MS_n: mass spectrometry tandem

n-BuOH: normal-butanol

n-hexane: normal hexane

NMR: nuclear magnetic resonance

NOESY: nuclear overhauser effect spectroscopy

NTS: Naturstoff reactive

PBS: phosphate buffered saline

PC3: human prostate cancer cell line

PEG: polyethylene glycol 4000

ppm: parts per million

Q-TOF: quadrupole- time of flight

RACI: Relative Antioxidant Capacity data Index

ROESY: rotating frame Overhause effect spectroscopy

RNA: ribonucleic acid

RP: reverse phase

SDA-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TLC: thin layer chromatography

TOCSY: total correlation spectroscopy

TPC: total polyphenolic content

TPPI: Time Proportional Phase Increment

TPTZ: 2,4,6-tripyridyl-s-triazine

t_R: retention time

UV: ultraviolet-visible

µg: microgramm

µM: micromolar

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